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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Determination of Vitamin D by a Chemical Method Involving Chromatography and Color Inhibition*

By J. B. WILKIE, S. W. JONES, and O. L. KLINE

A method for the determination of vitamin D applicable to pharmaceutical products is described in detail. The method involves saponification, extraction, chromatography, and color measurement by a modified antimony trichloride test. An important feature is the use of acetic anhydride which inhibits completely and selectively the vitamin D-$SbCl_3$ reaction, providing a reaction blank that greatly improves the specificity of the reaction. Of particular concern are the purity of color reagent, and the adsorption index of the magnesium oxide and alumina adsorbents used in the chromatography. Data are presented showing stability of vitamin D in the chromatography, the effect of acetic anhydride in the color reaction, and the validity of corrections based upon the acetic anhydride blank, and upon absorption differences at 500 and 550 mμ wavelengths. The character of the vitamin A degradation interferences is discussed and analytical results of the method applied to a series of pharmaceutical products are compared with bioassay values.

NUMEROUS ATTEMPTS to prescribe a means of estimating Vitamin D, either colorimetrically or spectrophotometrically, have, at best, been only partially successful, and the official methods for this vitamin continue to employ biological procedures. Several years' effort in this laboratory have resulted in refinements of previously described approaches that now permit application of the colorimetric method to pharmaceutical products of low potency. It is possible to carry out determinations with samples containing less than 1,000 U. S. P. units of vitamin D.

The color reactions of both vitamin D_2 and D_3 with $SbCl_3$ have been the basis for a number of studies that are comprehensively reviewed by Ewing, *et al.* (1). Among these, the work of Nield, *et al.* (2), showed improved sensitivity and reproducibility of the reagent with the use of acetyl chloride with chloroform. In later work from this laboratory, DeWitt (3) found that ethylene dichloride had a stabilizing effect when substituted for the chloroform solvent.

A different color reaction was described by Sobel, Mayor, and Kramer (4) who used glycerol 1,3-dichlorhydrin with acetyl chloride, and by Campbell (5) who further studied this reaction with vitamin D, improving sensitivity by selection of a suitable wavelength for color measurement.

Greene (6), in dealing with many phases of the vitamin D determination, developed an iodine trichloride reaction, but found it less sensitive and less specific than $SbCl_3$.

Several attempts to apply paper chromatography have been made (7–9). Experience with it in our laboratory indicated that separation was

* Received April 14, 1958, from the Division of Nutrition, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.

We are pleased to acknowledge the interest and help of a number of our colleagues who contributed in many ways to the progress of these studies. We particularly thank Mr. Glen Shue, Mr. Walter Morris, and Dr. Leo Friedman for their assistance.

We also acknowledge, with thanks to Dr. James Waddell of the Dupont Company, Dr. Norris Embree, Distillation Products, Inc., Dr. Saul Rubin, Hoffmann-La Roche, and Dr. Irwin Olcott of Dawe's Laboratories, the contribution of vitamin A or vitamin D preparations of specified purity that were invaluable in the development of these studies.

not sufficiently quantitative to serve as a basis for an analytical method.

Attempts to make use of direct spectrophotometric measurement of vitamin D even after chromatographic purification (3, 6, 10) have been applicable only to high potency materials. Ewing, *et al.* (11), in a later report, state that "for samples containing less than about 4,000 U. S. P. units of vitamin D per Gm. of oil the error begins to rise rapidly, and this value may be taken as a lower limit for the satisfactory determination of vitamin D in oil samples."

Thus the problem of applicability to pharmaceutical products resolves itself primarily to quantitative separation of vitamin D from interfering substances, principally vitamin A and its degradation products. Adsorption column chromatography of the nonsaponifiable fraction has been most useful for such separations. Ewing, *et al.* (11), employed a dual column procedure with superfiltrol separation followed by alumina. These workers observed that the interferences not removed by this chromatography were of two kinds. The first was a residue in the oil, and the second the decomposition product of vitamin A formed in the superfiltrol column.

Refinements in saponification, chromatography, and colorimetry have been the subject of extensive studies in this laboratory, and now provide a basis for workable procedures. The method described here allows a more quantitative separation of the vitamin A degradation products than previous methods. This separation problem cannot be avoided in providing a method useful in control programs since, in most pharmaceutical products, vitamin D is accompanied by vitamin A. Furthermore, vitamin A is useful as a guide in the chromatography. It has not been possible during chromatography entirely to prevent or eliminate vitamin A degradation products that have both chromatographic and spectral characteristics closely similar to those of vitamin D.

Specificity of the colorimetric step was greatly improved when it was observed that acetic anhydride quantitatively inhibits the color reaction of $SbCl_3$ with vitamin D measured at 500 mμ, but not the reaction with the major interfering substances which are principally vitamin A decomposition products.

PRINCIPLE OF METHOD

The method requires a sample containing approximately 1,000 to 2,000 U.S.P. units (25 to 50 mcg.) of vitamin D for the best precision, but one-half this amount can be determined by concentration of the final eluate fraction.

Saponification is carried out in alcoholic-KOH with 20 minutes' refluxing.

The nonsaponifiable fraction in petroleum ether is chromatographed, first on MgO that has been standardized with respect to its adsorptive power, for separation of vitamin D from vitamin A and extraneous substances. In a second chromatographing on alumina, vitamin D is separated from some of the fluorescent degradation products of vitamin A present in the sample or formed on the first column.

The vitamin D in the second column eluent is made to react with the acetyl chloride-antimony trichloride reagent to form a color, measured at 500 mμ by a procedure involving use of an internal standard. Acetic anhydride completely inhibits this reaction, providing a reaction blank, and an additional minor correction of the absorbance value depends upon a reading at 550 mμ, to eliminate any effect of decomposed vitamin A that may escape separation in the chromatography. The vitamin D reference solution in ethylene chloride is evaluated in each determination by multiplying the absorbance at 270 mμ by a factor that has been carefully determined.

METHOD

Reagents

Vitamin D Reference Solution.—Ethylene dichloride solution of crystalline calciferol or vitamin D_3. Prepare Stock Solution I to contain 25 mg. in each 100 ml. of petroleum ether or 10,000 units per ml. The Reference Solution is prepared by placing 1 ml. of Stock Solution I in a 50-ml. volumetric flask, removing the petroleum ether with a stream of nitrogen, then making to a volume of 50 ml. with ethylene dichloride, to contain 200 units of vitamin D per ml. or 5 mcg. per ml. All reference solutions are stored in the refrigerator.

KOH.—50% by weight, (780 Gm./liter).

Ethylene dichloride.—1,2-dichloroethane.

Cottonseed Oil.—Used as stabilizing agent of high antioxidant value, and must have a value of not less than 0.2 when measured in the following test: to an 18-mm. test tube add 0.2 ml. of 0.1% aqueous $FeCl_3 \cdot 6H_2O$, 1 ml. of 0.1% α,α-dipyridyl in absolute ethanol, and 5 ml. absolute ethanol. Shake, and after 5 minutes read absorbance at 520 mμ against absolute ethanol set at 100. Then add 0.1 ml. of a solution containing the nonsaponifiable fraction of 1 Gm. cottonseed oil per ml. in petroleum ether. After 5 minutes, read the absorbance, and obtain the absorbance value by subtracting the blank reading. Store in a refrigerator with minimum exposure to air.

Adsorbents—MgO.—SeaSorb 43 (Westvaco Co.) heated in a muffle furnace for three hours at 600°.

Alumina Hyflo-Supercel.—(Johns Manville industrial filter aid).

Alumina.—Alcoa grade F-20.

Treated Alumina.—Alcoa grade F-20. Generally the grading or mesh size distribution of this product is satisfactory. However, not more than 50% of the material should pass a 100-mesh sieve and the material passing the 100-mesh sieve but not the

160-mesh sieve should approximate 50%. The remainder should fall between 60- and 100-mesh. The selected alumina is heated in a muffle furnace for three hours at 600°, and after partial cooling, placed in a tightly closed screw-cap glass jar. After it reaches room temperature the material is sieved through an 80-mesh screen, weighed, then placed in a tared, screw-cap glass jar filled to not more than two-thirds capacity. Distilled water is added, dropwise, with frequent shaking of the capped bottle, until the material contains 3% added water. Shaking is continued for at least fifteen minutes so that no lumps remain, and the material is uniform. After standing overnight, tightly capped, the material when tested has an adsorption index within the range 30 to 40. Since a change in moisture content affects the adsorption index of the reagent, the container must be kept tightly closed, except when removing a portion for use.

Test for Adsorption Index of Alumina

Ten centimeters of the adsorbent to be tested is added and tapped into a settled position in a chromatographic tube 6 mm. (inside diameter) x 20 cm. plugged at its tip with glass wool. One milliliter of solution containing 20 mg./liter of the dye in petroleum ether (F & D Yellow No. 4 of about 99% purity) is then added to the top of the column. Sixteen per cent solution of U. S. P. ether in petroleum ether is then added in small portions from an accurately filled 50-ml. graduated cylinder, while five inches of vacuum is applied.

The number of ml. of this eluent to elute all of the dye is the adsorption index for the adsorbent under test. There may be some difficulty in determining the point at which all of the dye has been eluted from the column. This is accomplished by first collecting the eluate in a 50-ml. beaker until all apparent color in the column is eliminated, and finally by collecting 1-ml. fractions in 1-ml. beakers successively until, when viewed against a white background, the collected eluate is colorless. Removal of individual fractions is easily accomplished at 5-in. vacuum by first slipping the micro bell jar over the edge of the base plate. The total number of ml. to attain the colorless eluate is the required adsorption index.

Retentiveness is both a function of the adsorbed water and the condition of the anhydrous alumina itself. The adsorbed water can be removed by cautious heating at 600° for three hours. Weakened anhydrous alumina caused by too much heating or by heating over 600° should be avoided.

Ethyl Ether.—U. S. P. grade, peroxide-free ether is suitable for the extraction of the saponification mixture. For the second column chromatography, ether, redistilled over KOH pellets, is required. To this latter reagent are added pieces of bright copper strip, to inhibit peroxide formation. This reagent is checked frequently (U. S. P. test) and, at the first indication of peroxides, is discarded.

Petroleum Ether.—Must have a spectral transmission at 300 mμ of at least 85%, in a cuvette the light path of which is 1 cm., against a no-cell blank. Also, in the adsorbent activity test the eluent effect of 10 ml. of petroleum ether by itself must cause a movement of the visible color no more than 1 cm. below the surface of the column. To meet these requirements distillation may be necessary.

Eluting Solutions.—*First column:* 1. 0.5% absolute ethyl alcohol in petroleum ether, 2. 0.1% absolute ethyl alcohol in petroleum ether, 3. petroleum ether. *Second column:* 1. ethyl ether (redistilled over KOH), 2. 5% absolute ethyl alcohol in petroleum ether, 3. petroleum ether. *Test for Adsorption Index of Alumina:* 16% U. S. P. ether in petroleum ether.

Adsorbent Preparation W.—A weak adsorbent composed of 1 part MgO and 5 parts Hyflo-Supercel by weight, thoroughly mixed. When tested by the method described below it has an adsorption index of 5 to 10.

Adsorbent Preparation S.—A strong adsorbent composed of 1 part MgO and 1 part Hyflo-Supercel by weight, thoroughly mixed. When tested by the method described below it has an adsorption index of 20 to 35.

Test for Adsorption Index of MgO Mixtures

Pack tightly with a $^3/_{16}$ inch tamper, under 20 inches of vacuum, the mixed adsorbent to be tested into a 1.0 to 1.2-cm. tube fitted with a sealed-in fritted disk to a height of 1.5 cm. With the vacuum off, add 1 ml. petroleum ether solution containing 20 mg./liter F&D Yellow No. 4 of about 99% purity. Add about 2 ml. of 10% absolute alcohol in petroleum ether from an accurately filled 50-ml. graduated cylinder. Increase the vacuum to 20 inches and continue to add the 10% eluent until most of the dye band is eluted. Remove and discard the collected eluate. Collect successive 1-ml. fractions in 1-ml. beakers until, when viewed against a white background, the eluate is judged colorless. Removal of individual fractions is easily accomplished at 5-in. of vacuum by slipping the edge of the micro bell jar over the edge of its base plate. The number of ml. of 10% alcohol in petroleum ether to attain the first colorless eluate fraction is the adsorption index of the magnesium oxide mixture.

Antimony Trichloride Reagent.—*Solution A.*—Using antimony trichloride, U. S. P., in a dry, crystalline form, packed in a well-sealed glass-stoppered bottle, dissolve approximately 113 Gm. in sufficient ethylene dichloride to make 500 ml. To avoid moisture contamination, this is done quickly by emptying, without weighing, a previously unopened $^1/_4$-lb. bottle of antimony trichloride into about 400 ml. ethylene dichloride. Add approximately 2 Gm. anhydrous alumina, mix thoroughly, and filter the solution through filter paper into a clear glass reagent bottle, then make to a previously marked 500-ml. volume with ethylene dichloride. This solution is colorless, or nearly so, and must have a spectral transmittance of at least 85% at 500 mμ, measured against the ethylene dichloride solvent.

Solution B.—In a hood add 100 ml. acetyl chloride to 400 ml. ethylene dichloride with mixing, and store in a glass-stoppered bottle.

Color Reagent.—Mix, at least one-half hour before use, 45 ml. of Solution A and 5 ml. of Solution B, and keep in a glass-stoppered flask tightly stoppered until needed. This solution is satisfactory until it becomes colored. It is best to prepare it fresh on the day of use, although it may be useful over a period of a week.

Color Inhibitor.—A solution containing equal volumes of acetic anhydride and ethylene dichloride.

APPARATUS

Chromatographic Tubes

Tube for First Column Chromatography.—2.5 cm. in diameter x 20 to 30 cm. in length, with coarse fritted disk, and sealed to an 8 mm. x 5 cm. exit tube.

Tube for Second Column Chromatography.—6 mm. (inside diameter) x 20 cm. in length, with the lower 5 cm. pulled out to form a tapered constricted exit. About 1 cm. of the upper part of the constricted section is plugged with glass wool.

Tube for MgO Adsorption Index Test.—1 cm. in diameter by 10 cm. in length, with medium fritted disk, and sealed to an 8 mm. x 5 cm. exit tube.

Supplementary Evaporation and Chromatographic Apparatus

A vacuum micro bell jar large enough to hold a 100-ml. beaker is used in applying the vacuum and collecting the eluates. The vacuum either from the line or from a water vacuum aspirator is controlled by a vacuum gauge and a screw clamp bleeder. A stopcock or screw clamp bleeder on a T tube may be used to control the vacuum delivery to the gauge and chromatography assembly. Fifty- or 100-ml. beakers, a water vacuum aspirator attached to a vacuum gauge, and a three-way stopcock device with interchangeable florence flasks having standard glass joints may be used to expedite change of solvents (12). The use of manual automatic pipets is important because of the obnoxious or potentially harmful nature of the reagents and solvents used.

Ultraviolet Lamp.—A lamp source of weak ultraviolet is required for observing the fluorescent bands on the chromatographic column. The lamp should provide radiation in the longer (300 mμ) region. Suitable lamps may be constructed or are commercially available. For commercial lamps a narrow aperture or screen may be necessary to reduce the amount of destructive radiation.

Standardization of Test Tubes for Photometric Measurement

Photometrically matched tubes are required. A solution of F&D Orange No. 2 is particularly useful for this purpose.

Photometer.—Any direct reading photometer or monochrometor which has a sufficiently narrow absorption band at 500 and 550 mμ to provide linearity is suitable.

PROCEDURE

Sample

A desirable size of sample is one that contains 1,000 to 2,000 units of vitamin D. A smaller amount can be determined by reducing the volume of the final solution used for color measurement. However, the concentration of the final solution should not fall below 50 units per ml. A condition to be observed also is the amount of vitamin A contained in the sample. If the sample contains little or no vitamin A, approximately 3,000 units are added to provide the pilot bands necessary in the chromatography. However, the total amount present must not exceed the capacity of the first adsorption column which is usually in the range of 20,000 to 25,000 U. S. P. units of vitamin A.

Capsules or tablets are disintegrated by adding 10 ml. H_2O, warming on a steam bath with the 200-ml. Erlenmeyer flask at an angle, for ten minutes. It may be necessary to crush them with a flattened glass rod, and to warm for an additional five minutes. A metal clamp on the neck of the flask is useful for holding the flask at an angle, and for holding it immersed in the steam bath during the subsequent saponification step.

Saponification

To a measured amount of sample in a reflux flask add 1 ml. cottonseed oil, and 2.5 ml. of 50% KOH for each Gm. of sample taken, but not less than 15 ml. Then add 50 ml. of 95% ethanol for samples of less than 5 Gm. For samples of between 5 and 10 Gm. of fat or oil, add 75 ml. of 95% ethanol. Boil vigorously twenty minutes (thirty minutes for 5 to 10-Gm. samples of fat or oil) immersed in a steam bath, with a suitable reflux condenser. Cool the saponification container. Transfer contents to a 500-ml. separatory funnel, rinsing with 50 to 70 ml. of distilled water in several portions, adding each portion to the separatory funnel. Then add 50 to 100 ml. of ethyl ether. Good separation and relative freedom from emulsions at this step are obtained if the volumes of water, alcohol, and ether are maintained in approximately a 1:1:1 ratio. Shake vigorously and allow to stand about two minutes.

Separate the aqueous layer into another 500-ml. separatory funnel, and extract it three more times with 30-ml. portions of ether, adding each ether layer in turn to the original ether extract. In the case of slow separation or emulsion formation, add 2 to 5 ml. of 95% alcohol and swirl gently. Solid material in the aqueous phase is forced through the funnel with a wire of suitable size.

Pour 100 ml. of distilled water through the combined ether extracts, wait five or more minutes for complete separation, and discharge the lower water layer into another separatory funnel. For 10-Gm. oil samples, 200 ml. of water may be necessary here. This aqueous fraction is then extracted twice with 50-ml. portions of ether and the resulting layers added to the original ether extract. These two ether extractions should be accomplished with vigorous shaking. Good separations will generally require subsequent gentle swirling with additions of about 5 ml. of ethanol. Allow two to five minutes for separations in this step. Pour two 100-ml. portions of distilled water through the combined ether extracts without shaking, allow to separate, and discard these aqueous fractions. Add about 10 ml. of water and shake vigorously. If there is a slight persistent emulsion formed at the interface, dilute by pouring water through the ether extract, and discard the aqueous layer.

Where particularly persistant emulsion occurs, or where precipitates gather at the interface, gather as much as possible of this material at the interface by shaking with about 3-ml. volumes of water or dilute alkali solution. Isolate the interface material in a separatory funnel set aside for this purpose. It is important to collect in one funnel all of the separations of such interface material even though some of the solvent portions are unavoidable. This interface fraction is then shaken with about 25 ml. of petroleum ether which will likely become cloudy.

The cloudiness is removed by swirling with about 3 ml. of 95% alcohol. The clear petroleum ether solution is then added to the original solvent extract, and in like manner, the interface fraction is extracted two more times with 25 ml. of petroleum ether and 3 ml. of 95% alcohol, adding each to the original ether extract.

Add successive portions of distilled water to the ether extract with gentle agitation, removing each until the rinse water is free of alkali as shown by a phenolphthalein test. Drain off the last small fraction of water and discharge ether extract into a 300-ml. tall beaker containing about 5 Gm. anhydrous Na_2SO_4. Stir for about two minutes, and transfer the ether extract with ether rinsing, to another 300-ml. tall beaker. Evaporate the extract to about 30 ml. on a steam bath and transfer to the flask of the evaporation apparatus for final evaporation under vacuum.

The evaporation flask is placed in the steam bath until a volume of 5 ml. remains. The flask is then placed under vacuum in a water bath at a temperature below 40° until evaporation is complete, as indicated by appearance and an increase in vacuum. Introduce petroleum ether, dissolve the residue, and make to a volume of 10 ml. This is the sample extract.

First Column Preparation.—Using a rod approximately 1/4 inch in diameter, tamp W adsorbent tightly into the large chromatographic tube, under 25 inches of vacuum, to form a column segment 2.5 cm. in height; make the surface level and smooth.

Under the same vacuum add S adsorbent to a height of approximately 10.5 cm. With the surface level, press the material firmly with a cork attached to a glass rod so that this section is about 8 cm. in height. The cork should have approximately the same diameter as the tube, fitting loosely, to provide even pressure throughout the cross section of the tube.

Complete the column by the addition of a layer of about 1 cm. of anhydrous Na_2SO_4.

First Column Chromatography—Sample Addition.—With the column under 5 inches of vacuum, pour 30 ml. of petroleum ether on the column, slowly down the side of the slightly tipped tube. When 20 ml. of petroleum ether remain above the Na_2SO_4 layer, release the vacuum and add the sample extract, again pouring carefully down the side of the slightly tipped tube. Apply 20 inches of vacuum and, as the surface of the liquid disappears into the Na_2SO_4 layer, reduce the vacuum to 5 inches and quickly add 3 small portions (2 to 5 ml.) of petroleum ether down the side of the tube, allowing each portion to move into the Na_2SO_4 layer before adding the next. Then carefully add 25 ml. petroleum ether. As the surface of this addition reaches the Na_2SO_4 layer, add the eluting solution containing 0.1% alcohol in petroleum ether in 10-ml. portions. In the period following the first addition of this eluent, the fluorescent vitamin A begins to move in a compact band, and separating ahead of it is a narrow yellow fluorescent band. The movement of these bands can be followed by frequent brief inspections with the ultraviolet lamp. At the end of fifteen minutes increase the vacuum to 20 inches; and by the end of 30 minutes, the fluorescent bands should move one-quarter to one-half the length of the upper segment of the column.

If the lower band has moved one-half the length of the upper segment in less than fifteen minutes, its movement is retarded by substituting petroleum ether for the 0.1% alcohol eluent, and by keeping the vacuum at 5 inches throughout the elution. (With some experience it is possible to make use of a chromatogram that develops rapidly whenever there is a 2 to 3-cm. separation between the bands. In such case, increase the vacuum to 20 inches, change the collection flask when the yellow fluorescent band reaches the junction, and change to petroleum ether for eluting when the vitamin A band is between 1 and 2 cm. from the junction.)

If the bands have failed to move as described above with the vacuum at 5 inches, add 5 ml. of the 0.5% alcohol in petroleum ether eluent, followed by 25 ml. of the 0.1% eluent, repeating this sequence of additions if necessary, until movement is apparent, and the band has moved one-half the length of the upper segment. Then increase the vacuum to 20 inches.

At this point the separation of the two fluorescent bands should be greater than 1 cm. and when the lower yellow fluorescent band reaches the junction of the two adsorbents, the eluate is discarded and a clean collection beaker is placed in the bell jar.

Elution is continued by the addition of the 0.1% alcohol eluent until the vitamin A band reaches a point 1 cm. above the junction, then petroleum ether alone is used as the eluent until the junction is reached.

When the vitamin A band reaches the junction, the vacuum is reduced to 5 inches, and elution is continued with petroleum ether until the lowest tip of the vitamin A band touches the fritted disk. At this point collection is discontinued, using great care to collect as much eluate as possible without including any vitamin A.

The stem of the chromatographic tube is rinsed with petroleum ether into the collection beaker, and the eluate is transferred to the evaporation apparatus.

The evaporation flask is placed in the steam bath and evaporation continued until a volume of about 5 ml. remains. The flask is then placed in a water bath at a temperature below 40°, under vacuum, until evaporation is complete, as indicated by appearance and an increase in vacuum. Introduce petroleum ether, dissolve the residue, and make to a volume of 5 to 10 ml. This eluate solution is used for the second column chromatography.

Second Column Preparation.—Add to the small chromatographic tube 20 cm. of treated alumina, avoiding extending the column into the enlarged section of the tube. The column is made sufficiently compact by tapping the side of the tube during and after the addition of the adsorbent.

Second Column Chromatography.—With the column under 20 inches of vacuum, and a 25-ml. collecting beaker in place, add the eluate solution from the first column, rinsing the flask with a small amount of petroleum ether. As soon as the eluate solution has moved into the column, reduce the vacuum to 5 inches, and add 2.0 ml. of reagent ethyl ether. When this has moved into the column add 7 ml. of petroleum ether and allow it to move into the column. Release the vacuum and remove the eluate, identifying it as fraction No. 1. With a clean 10-ml. collection beaker continue elution,

under 5 inches of vacuum, with the addition of 20 ml. of 0.1% alcohol eluent. Three separate eluate fractions are collected, each approximately 5 ml. and identified as fractions No. 2, No. 3, and No. 4. These fractions are precautionary to make certain that the chromatography is effective. Normally, when these fractions are exposed to the weak ultraviolet light, fraction 1 fluoresces strongly, fraction 2 may show some, and fractions 3 and 4 are without fluorescence. If fraction 3 is nonfluorescent, and fraction 4 shows fluorescence, then the latter is added to fraction 5 which is fluorescent. But if all fractions show fluorescence, the chromatography has been inadequate, and should be repeated. For collection of eluate fraction No. 5 in a 50-ml. collection beaker, 25 ml. of 5% alcohol eluent is added with the vacuum at 20 inches.

Evaporate the eluate fraction No. 5 in a 50-ml. flask in the evaporation apparatus. The evaporation flask is placed in the steam bath until a volume of 5 ml. remains. The flask is then placed in a water bath at a temperature below 40°, under vacuum, until evaporation is complete, as indicated by appearance and an increase in vacuum. Alternately, the eluate fraction may be evaporated on a steam bath to a volume of about 2 ml. The beaker is then removed from the steam bath and evaporation is completed under a light stream of nitrogen. Introduce 5 ml. of ethylene dichloride, dissolve residue, and transfer to a 10-ml. volumetric flask, rinsing with several small portions of ethylene dichloride. Make to a final volume of 10 ml. This is the sample solution to be used in the color reaction.

Color Reaction.—All colorimetric measurements are made in the same tube or in matched tubes. The tubes must be rinsed with ethylene dichloride three times after each use. Exactly thirty seconds after the addition of the color reagent, the transmittance reading of 500 mμ is taken with the instrument set at 100% transmittance with ethylene dichloride. A second reading is made at 550 mμ within ninety seconds after the color reagent addition. An internal standard is used to compensate for possible sample effects on color development. The color reagent is added rapidly. For this, a device such as a 10-ml. graduated cylinder cut off at the 7-ml. mark is practical, or an automatic measuring pipet may be used. The three color reactions are carried out in the following order: (*a*) 1 ml. sample solution + 1 ml. reference solution + 5 ml. color reagent. (*b*) 1 ml. sample solution + 1 ml. ethylene dichloride + 5 ml. color reagent. (*c*) 1 ml. sample solution + 1 ml. color inhibitor + 5 ml. color reagent.

Standardization of Reference Solution.—The vitamin D reference solution in ethylene chloride is evaluated in each determination by measuring its absorbance at 270 mμ. Concentration of vitamin D, in units per ml., is calculated:

$$A_{(r)\ 270} \times 860 = \text{units per ml.}$$

Calculation.—Convert transmittance readings to the corresponding absorbance values, then with:

$A_{(s+r)\ 500}$ = 500 mμ absorbance of sample solution + reference solution

$A_{(s)\ 500}$ = 500 mμ absorbance of sample solution

$A_{(s+i)\ 500}$ = 500 mμ absorbance of sample solution + color inhibitor

$A_{(s)\ 550}$ = 550 mμ absorbance of sample solution

$A_{(s+i)\ 550}$ = 550 mμ absorbance of sample solution + color inhibitor

$$A_{REF} = A_{(s+r)\ 500} - A_{(s)\ 500}$$

$$\frac{\text{Reference concn. (units/ml.)}}{A_{REF}} = K \text{ (Concn. of vitamin D per unit of absorbance)}$$

$$[(A_{(s)\ 500} - A_{(s+i)\ 500}) - 0.67\,(A_{(s)\ 550} - A_{(s+i)\ 550})] = A\ (\text{corr. } S)_{500}$$

$A\ (\text{corr. } S)_{500} \times K$ = vitamin D content of sample solution (units/ml.)

Units per ml. sample solution/C = vitamin D content of sample, where C is the concn. in Gm., capsules, or tablets per ml. of colorimetrically evaluated sample solution of the material taken for analysis.

DISCUSSION

Preparation of Sample.—Although oils are readily saponified there is advantage in treating capsule or tablet samples with a small amount of hot water to speed disintegration and to reduce the time necessary for complete saponification. With such treatment of oil-insoluble coatings the twenty-minute saponification time is generally adequate.

Vitamin D has been shown to be unstable on the MgO chromatographic column, in the absence of antioxidant and samples of unknown composition that do not contain antioxidant have been encountered. The addition of 1 ml. fresh cottonseed oil to each sample assures stability of the vitamin throughout the procedure. Results of comparison of vitamin D chromatographed with and without addition of cottonseed oil are shown in Table I.

TABLE I.—RECOVERY OF VITAMIN D FROM CHROMATOGRAPHIC COLUMN IN PRESENCE AND ABSENCE OF ANTIOXIDANT

Without Cottonseed Oil			With Cottonseed Oil[a]		
Vitamin D Added, Units/ml.	Vitamin D Recovered, Units/ml.	Recovery, %	Vitamin D Added, Units/ml.	Vitamin D Recovered, Units/ml.	Recovery, %
150	129	86	200	192	96
200	176	88	200	195	98
200	114	57	200	192	96
200	128	64	200	191	96
			200	193	97

[a] 1 ml. added to each sample.

Saponification.—Immersion of the saponification flask in a steam bath is prescribed. Incomplete saponification leads to difficulties in control of the chromatography. An efficient reflux condenser over a flask heated on a hot plate may cool and slow the process to such an extent that saponification is not completed in the twenty-minute period. In such a case the presence of a small amount of fat in the solution for chromatography causes rapid elution of the visible bands and prevents the necessary separation of vitamins A and D.

Extraction.—The procedure described is essentially that used for fish-liver oils in determining vitamin A content by the A. O. A. C. method. The additional treatment required for samples in which resistant emulsions or precipitates at the solvent interface occur has been found quite satisfactory in practice. However, relatively few samples that require such treatment have been encountered.

Chromatography.—The chromatographic procedure is the foundation of this method and the culmination of long experience and many experiments. Important improvement resulted from the knowledge that sufficient uniformity may be assured by tempering the MgO with heat and Hyflosupercel, and the alumina adsorbent with heat and water. Adsorptive capacity of the alumina is maintained within a narrow and workable range by heating it at 600° for three hours, then adjusting the moisture content to 3%. Assurance of suitability of the adsorbents used by determining adsorption index is essential. Also, particle size of the alumina is important.

Most pharmaceutical samples containing vitamin D also contain vitamin A. Since the method described here depends upon the vitamin A and yellow fluorescent pilot bands, a measured amount of vitamin A must be added to those samples devoid of it. Separation of all vitamin A is accomplished on the first column. To assure this separation it is important that a good division is obtained between the vitamin A band and the yellow fluorescent band that moves ahead of it. In this case, the vitamin D moves well ahead of the vitamin A, but is too close to the yellow fluorescent band to permit adequate separation of these two on this column. The fraction eluted ahead of the yellow band eliminates unidentified interference, probably sterol in nature. Great care and some skill are necessary in carrying out this chromatography successfully. There is evidence that the yellow fluorescent band material contains both colored and colorless fluorescent substances. Further evidence indicates that these substances arise largely as degradation products of vitamin A. For example, with repeated chromatography of the vitamin A band on the MgO column, additional yellow fluorescent band material appears in approximately the same proportion in each separation preceding the vitamin A band. We have observed this as well when starting with vitamin A of high purity. It is usually not difficult to separate the yellow material from vitamin D in the alumina column. It has not been possible, however, completely to eliminate a colorless fluorescent substance from the vitamin D fraction, a substance which has spectral absorption in the ultraviolet closely similar to that of vitamin D, but which causes little interference in the $SbCl_3$ color reaction. Because of an inability to accomplish this complete separation, determination of absorbance of the vitamin D eluate fraction at 270 mμ is not a reliable measure of vitamin D.

In Fig. 1 are shown absorption curves for vitamin D_3 in ethylene chloride (Curve A) and in ethyl alcohol (Curve B). The yellow fluorescent fraction that is separated from the vitamin D fraction in the second column has the absorption characteristics shown in Curves C and D. The vitamin D eluate fraction, Curve E, shows characteristic absorption in the 270 mμ region, but is displaced to the right at the higher wavelengths. The absorption of vitamin A epoxide, as described by Karrer (13), is represented by Curve F. That vitamin A epoxide, or a degradation product of vitamin A having closely similar absorption characteristics, is the contaminant of the vitamin D eluate fraction from the second column chromatography is demonstrated in Fig. 2. A sample of vitamin A, free of vitamin D, was taken through the chromatography, and absorption of the fraction that would be expected to contain the vitamin D was measured. This is shown as Curve C. Again Karrer's vitamin A epoxide is included as Curve B, and for reference Curve A is the absorption of vitamin D in ethylene chloride. It is quite clear that the chromatography used does not provide a separation sufficiently complete to rely upon direct spectrophotometric absorption of the final eluate as an estimate of the vitamin D present.

The procedure for the $SbCl_3$ vitamin D color reaction is essentially DeWitt's modification (3) of Nield's reaction. No other color reaction with vitamin D has been found to equal the sensitivity of that with the $SbCl_3$ acetyl chloride reagent. It is quite susceptible to interference, however, where vitamin A or its degradation products are present. We have learned that in the presence of acetic anhydride the color reaction with vitamin D is completely inhibited, while that with the common interfering substance is inhibited to a small extent or not at all.

The effect upon vitamin D alone is shown in Table II.

TABLE II.—EFFECT OF ACETIC ANHYDRIDE UPON THE VITAMIN D_3-$SbCl_3$ REACTION

Units, Vitamin D/ml.	Absorbance at 500 mμ Without Acetic Anhydride	With Acetic Anhydride
100	0.120	0.001
200	0.237	0.000
400	0.488	0.001

TABLE III.—APPLICATION OF ACETIC ANHYDRIDE INHIBITION TO SPECTROPHOTOMETRIC EVALUATION OF VITAMIN D-$SbCl_3$ COLOR

Sample	1. Total Absorbance at 500 mμ	2. Absorbance with Acetic Anhydride	3. Absorbance Correction from 550 mμ Reading	4. Vitamin D Absorbance
1	0.272	0.076	0.015	0.181
2	0.196	0.043	0.005	0.148
3	0.161	0.051	0.017	0.093
4	0.268	0.078	0.018	0.172

In Table III are given examples of absorbance values from Beckman Spectrophotometer Model B readings of sample solutions prepared from four different samples. In the description of the method these readings correspond as follows:

Column 1 is $A_{(2)}$ 500; column 2 is $A_{(2+4)}500$; column 3 is $0.67(A_{(2)}550 - A_{(2+4)}550)$; column 4 is column 1 minus the sum of columns 2 and 3.

It is clear that the total absorbance values shown in column 1 do not accurately represent the vitamin D present. The interference absorbance shown in column 2 has been an important source of error in earlier work. The correction shown in column

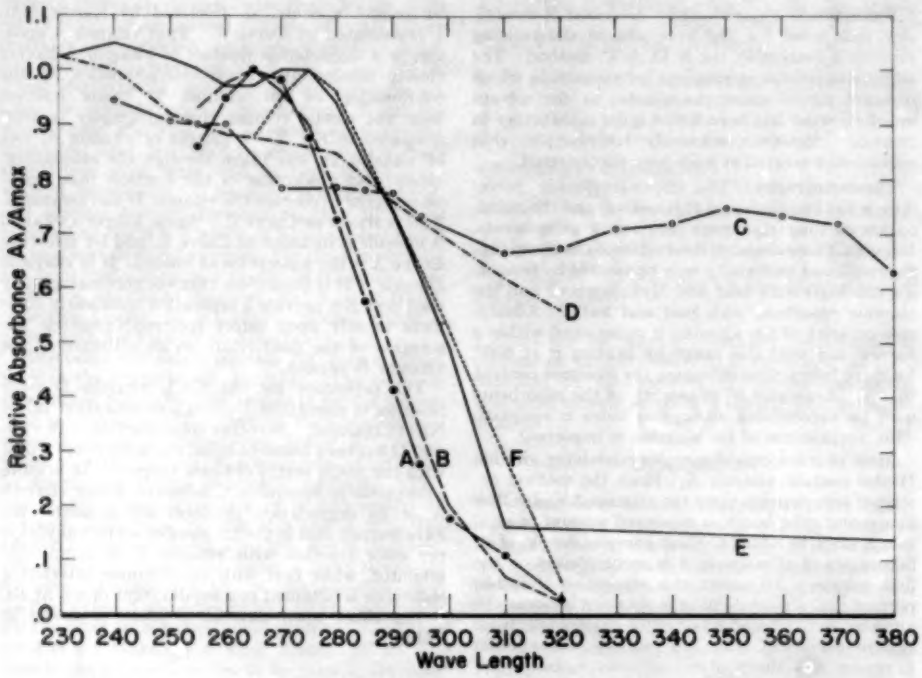

Figure 1.

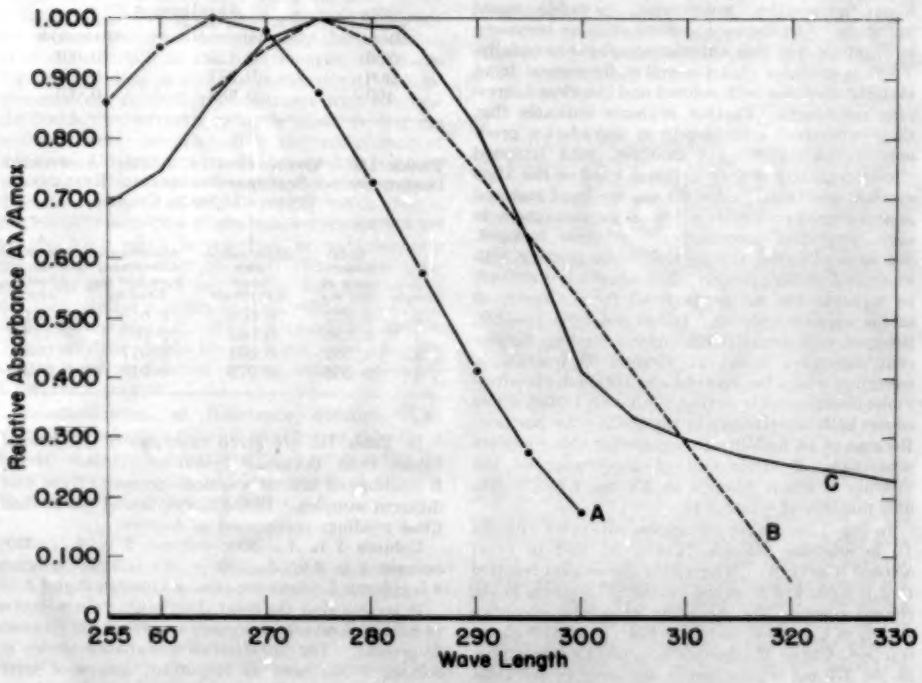

Figure 2.

3 is small and frequently negligible with the chromatography used.

Basis for the small correction indicated in column 3 is given in Table IV, the data for which were obtained as follows.

The method was applied to four samples that contained vitamin A, but no vitamin D. In the color test of the final eluate, the so-called vitamin D eluate fraction from the second column, the absorbance values for the sample (Tube $A_{(3)}$), and the sample plus acetic anhydride (Tube $A_{(3+5)}$) were determined at 500 and at 550 mμ.

The difference between tubes $A_{(3)}$ and $A_{(3+5)}$ at each wavelength are expressed as Δ absorbance, shown in Table IV. The ratio of the differences is 0.67, which is in close agreement with a large number of such determinations. Since the vitamin D-$SbCl_3$ color has no absorption at 550 mμ, it is possible to correct for non-vitamin D color by subtracting from the total absorbance, two-thirds of the difference observed at 550 mμ. This is the correction shown in column 3 of Table III. Nield (2) and Greene (6) attempted a correction based upon the 550 mμ absorption. This non-vitamin D absorption seldom exceeds 10% of that of vitamin D. When the correction is excessive a rechromatographing of the final eluate is desirable.

In color measurements of solutions containing from 50 to 1,000 units of vitamin D per ml. of final solution, Beer's law was found to apply. The smallest deviations were observed between 100 and 400 units per ml., and it is recommended that final concentration be kept within these limits.

TABLE IV.—Δ ABSORBANCE VALUES AT TWO WAVELENGTHS FOR THE VITAMIN D ELUATE FRACTION

Sample	Δ Absorbance at 500 mμ	Δ Absorbance at 550 mμ	A_{500}/A_{550}
1	0.019	0.029	0.66
2	0.025	0.036	0.70
3	0.018	0.025	0.72
4	0.021	0.035	0.60
		Average	0.67

The $SbCl_3$ reagent, Solution A, has been found to cause difficulty if its transmittance of 500 mμ falls below 85%. An occasional bottle of the solid $SbCl_3$ is found to contain color which, in solution, absorbs in the critical region. Also, moisture present in Solution A may cause a cloudiness that alters transmittance. Solution A is usually stable after the treatment with anhydrous alumina, but should be checked spectrophotometrically at frequent intervals. Any indication of change requires additional treatment with the anhydrous alumina. If color is excessive, it is necessary to prepare fresh reagent. Color change is readily noticed when the solution is stored in a clear glass bottle.

Since the color reaction is affected by substances other than vitamin D contained in the final sample solution, the use of an internal standard is essential. Variation between aliquots and from sample to sample, in the relation of absorbance to vitamin D, is shown in Table V. The values shown are the ratio of units of vitamin D per ml. of final solution to absorbance value. This is the K value obtained in the calculation of results.

TABLE V.—VARIATION IN K RATIOS OF VITAMIN D TO ABSORBANCE VALUE IN THE COLOR TEST

For Aliquots of Final Solution of a Single Sample	For Final Solutions from Separate Chromatographings of a Single Sample	For Final Solutions of Different Samples
1140	880	989
1070	1022	985
1150	1065	824
1050		1015
Coeff. of var. 4.45	5.2	14.3

It is apparent that there is a greater variation of K between samples than between aliquots of the same sample. Error from these variations is controlled by the use of the internal standard.

TABLE VI.—VITAMIN D CONTENT OF SEVERAL PHARMACEUTICAL PREPARATIONS

Sample	Label Declaration (U.S.P. Units)	Analysts' Results (U.S.P. Units) Analyst A	Analyst B	Bioassay Results (U. S. P. Units)
1. Elixir	1,300 units/5 ml.	1,450	1,450	1,950
2. Syrup	533 units/ml.	605	600	675
3. Liquid	2,000 units/5 ml.	1,345	1,315	1,500
4. Tablet	2,500 units/tab.	3,080	2,910	3,200
5. Capsule	1,000 units/cap.	1,430	1,005	1,140
6. Capsule	2,500 units/cap.	2,740	3,070	3,150
7. Yeast	...	78,875	...	75,000
8. Syrup	1,000 units/0.6 ml.	1,100	1,050	1,280
9. Capsule	1,200 units/cap.	1,320	1,150	1,140

APPLICATION

Although the method in its various stages of development has been used in this laboratory over a period of several years, and has been applied to a variety of products, the most effective use has been in the category of pharmaceutical preparations. The list shown in Table VI illustrates the types of material most successfully analyzed. Also from the results of two independent analysts the uniformity of results can be judged. The analytical values are compared with the bioassay result for each sample listed. The bioassays were not conducted specifically as a part of this study, but results were obtained as part of the routine assay program of the laboratory. It is clear that the results of the two methods are in the same range.

We have had little experience in applying the method to foods containing vitamin D. Here the problem is to saponify and extract sufficient material to provide approximately 1,000 to 2,000 units of vitamin D. With a suitable extract there would appear to be no great difficulty in extending the method to foods.

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An Identity Test for Iodinated Derivatives of Tyrosine and Thyronine, Particularly Triiodothyronine and Thyroxine*

By PAUL Z. ANTHONY and JAMES E. GEEVER

The Kendall-Osterberg color reaction for thyroxine has been applied to other iodinated phenolic amino acids. It provides a ready means of identifying individual members of two families of compounds.

A RAPID METHOD for distinguishing between the various iodinated derivatives of tyrosine and 3,5-diiodothyronine has been developed using the method of Kendall and Osterberg (1). In their work on the chemical identification of thyroxine these investigators noted that thyroxine gives a red color when treated first with nitrous acid and then with ammonia. Ingvaldsen and Cameron (2) found that this color reaction occurs with both diiodotyrosine and thyroxine. They noted that the color is given by a phenolic compound containing two iodine atoms *ortho* to the hydroxy group; they found that the color is not given by dibromotyrosine, nor by a large number of other benzene derivatives. Morton and Chaikoff (3) adapted the reaction to a photometric determination of thyroxine and diiodotyrosine in thyroid tissue. Roche and Michel (4–6) conducted a detailed study of the reaction in the analyses of thyroid gland and iodinated proteins. Adamson, *et al.*, (7), used the reaction for the determination of synthetic sodium L-thyroxine, particularly in tablets containing thyroxine as the active ingredient in microgram quantities. Gross and Pitt-Rivers (8) used 40% sodium hydroxide instead of concentrated ammonium hydroxide to obtain a brownish-orange color with both thyroxine and 3,5,3′-triiodothyronine; the solution had a maximum absorption between 460 and 470 mμ.

In our laboratory the Kendall-Osterberg reaction gave characteristic colors for iodo-derivatives of tyrosine and 3,5-diiodothyronine.

EXPERIMENTAL AND RESULTS

The optimal concentration of an iodinated compound for color development under the conditions of the test is 0.045 to 0.050 microequivalents per cc. of final solution. The test is run as follows using 3,5,3′-triiodothyronine as an example.

A 20-mg. portion of L-triiodothyronine hydrochloride (0.03 milliequivalent) is dissolved in 50 cc. of 95% ethanol (wherever an alcohol-insoluble amino acid is used, a few drops of dilute hydrochloric acid are added to bring about solubility). A 2-cc. aliquot is placed in a 25-cc. volumetric flask and diluted with 4.5 cc. of 95% ethanol and 12.2 cc. of acidic sodium chloride reagent (170 Gm. of sodium chloride in sufficient 1 *N* hydrochloric acid to make 1000 cc.). A 2.5-cc. portion of freshly prepared 1% sodium nitrite solution is added, and the resulting solution placed in the dark for twenty minutes. A yellow color develops. Addition of approximately 3.8 cc. of concentrated ammonium hydroxide (up to the graduated mark) produces an orange color.

This test was also performed on dihydroxyphenylalanine and tryptophan and the results, as well as results on various iodinated phenolic amino acids, are listed in Table I.

Spectrophotometric absorption curves of the colored products of triiodothyronine and thyroxine exhibited absorption maxima at 475 and 495 mμ, respectively (Fig. 1). This distinct difference in the maxima showed that the orange color of triiodo-

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thyronine is not a dilution of the pink color of thyroxine but is characteristic of triiodothyronine.

DISCUSSION

Roche and Michel (5) reported that, in addition to thyroxine and diiodotyrosine, the reaction is given by three noniodinated amino acids, dihydroxyphenylalanine, tryptophan, and tyrosine. Tryptophan gives a measurable color only in a much greater quantity (1 mg.) than would be present in samples used in their method, and tyrosine gives only about $^1/_{80}$ the color intensity of diiodotyrosine. They also reported that monoiodotyrosine does not give a color with nitrous acid and ammonia. Contrary to their findings we found that: (*a*) dihydroxyphenylalanine gives an amber color; (*b*) tryptophan gives no observable color even in greater quantity (ten times optimal concentration); (*c*) tyrosine gives no observable color, and (*d*) monoiodotyrosine gives an orange color. Further investigation of the color obtained with dihydroxphenylalanine showed that the same color is produced when the amino acid is dissolved in concentrated ammonium hydroxide alone. Therefore, the coloration of this substance is probably due to formation of a quinoid structure under conditions of alkaline oxidation and not to any reaction with nitrous acid. During the addition of the nitrous acid no color formation was observed, whereas a yellow color is always obtained with iodinated tyrosines and 3,5-diiodothyronines.

Table I.—Kendall-Osterberg Color Reaction with Aromatic Amino Acids

Amino Acid[a]	Color
Tyrosine	Colorless
Monoiodotyrosine	Orange
Diiodotyrosine	Pink
3,5-Diiodothyronine	Colorless
3,5,3′-Triiodothyronine	Orange
Thyroxine	Pink
Dihydroxyphenylalanine	Amber
Tryptophan	Colorless

[a] The first 6 compounds were prepared analytically pure in our laboratory. The dihydroxyphenylalanine was Eastman's white label grade. The tryptophan was an analytically pure compound obtained from Schwarz Laboratories, Inc.

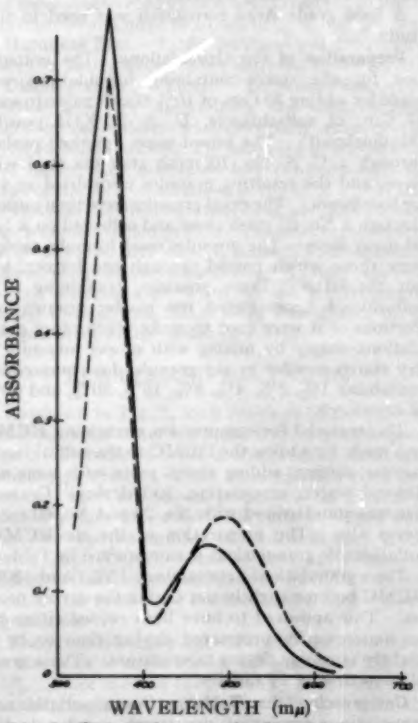

Fig. 1—Spectrophotometric absorption curves of the colored products of triiodothyronine and thyroxine. Concentration: 4.5×10^{-5} *M*. Triiodothyronine: ———; thyroxine - - - - -.

Besides using this method for the determination of thyroxine in tablets, Adamson, *et al.* (7), found that 3,5-diiodothyronine gives about 65% of the color intensity of thyroxine when subjected to the color reaction. On the other hand, we obtained no color with diiodothyronine. Since our method is a virtual duplication of theirs, this difference is not attributable to experimental conditions. It may involve compound purity, and in this connection, it is noteworthy that 3,5-diiodothyronine and tyrosine are similar in that no iodine is present on the hydroxylated benzene ring; therefore, no reaction with nitrous acid would be expected.

Plati and Wenner (9) used this color test to determine the completeness of extraction of 3,5,3′-triiodothyronine from a crude triiodothyronine containing 10–15% thyroxine as an impurity in a continuous countercurrent solvent extraction column. They reported a red color as a positive test for 3,5,3′-triiodothyronine. This is in disagreement with our results shown in Table I. We obtained an orange color corresponding to the orange color obtained with monoiodotyrosine, a molecule of related structure. It appears that the orange color is associated with an *ortho* iodo-phenolic hydroxyl relationship on an aromatic amino acid. It is predicted that 3′-iodothyronine, 3,3′-diiodothyronine, or any 3′-iodinated thyronine will give the same color.

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Properties and Applications of Powdered Polysaccharide Acids IV.*

Comparison of Carboxymethylcellulose (HCMC) and Cornstarch as Tablet Disintegrants

By LLOYD KENNON† and JOSEPH V. SWINTOSKY†

Sulfathiazole tablets were prepared through a compressional force range of 0.25 to 2 tons per tablet. Tablet hardness and disintegration time were compared for carboxymethylcellulose and cornstarch disintegrants through a concentration range of 1 to 30%. The results indicate that hardness and disintegration times were similar for both disintegrants, except at the 30% starch concentration where tablet hardness was very low. Generally, hardness increased rapidly with increase in compressional force and was not depressed appreciably by increased disintegrant concentrations. Disintegration times decreased rapidly with increased disintegrant concentrations. The principal advantages of HCMC in this formulation are that it can be granulated wet with the other tablet components without losing its disintegrant properties upon drying; also, wet granulations employing it dry faster than those without it, and the resultant tablets are somewhat harder than the corresponding tablets containing starch as the disintegrant.

IN A PREVIOUS PUBLICATION the preparation and properties of carboxymethylcellulose (HCMC) obtained by ion exchange of sodium carboxymethylcellulose followed by spray drying were described (1). Possible areas of pharmaceutical usefulness of this product have been noted (2–4). The present study was undertaken to compare the disintegrant properties of this material to cornstarch in sulfathiazole tablets. An effort was made to determine the interrelationships of such variables as compressional force, hardness, disintegrant concentration, and disintegration time.

The literature describes a comparison of potato starch and alginic acid as disintegrating agents (5). Also, a number of publications have defined the problems, described the equipment, and suggested methods for studying the static and dynamic factors which affect tablet formulation and manufacture (3, 5–27).

EXPERIMENTAL

Source of HCMC and Starch.—The method of preparation of the HCMC by ion exchange was modified from the previous procedure (1). Eight 4,000-cc. Erlenmeyer flasks, each containing 1 Kg. of acid-charged sulfonic acid cation exchange resin, Amberlite IR-120 (Rohm & Haas, Philadelphia, Pa.) were employed. Three liters of 2% CMC, Premium Low Viscosity-Type 70 (Hercules Powder Co., Wilmington, Delaware), in aqueous solution was added to flask 1, shaken periodically for fifteen to twenty minutes, then decanted into flask 2 after the resin settled. The process was repeated serially until transfer through the eight flasks was complete. About 10 liters of solution were made before it was necessary to wash and recharge the resin. A white powder was obtained by spray drying the HCMC solution as previously described (1).

A food grade Argo cornstarch was used in this study.

Preparation of the Granulations.—The granulation for the starch-containing formulations was made by adding 30 Gm. of 10% starch paste to each 97 Gm. of sulfathiazole, U. S. P. XIII powder (Mallinckrodt). The mixed mass was then pushed through a U. S. No. 10 mesh stainless steel wire sieve, and the resulting granules were dried at 45° for four hours. The dried granules were then pushed through a No. 20 mesh sieve and collected on a No. 60 mesh sieve. The granules used to make tablets were those which passed through the former, but not the latter. These granules, containing 97% sulfathiazole, constituted the master granulation. Portions of it were used to make seven other granulations simply by mixing with proper amounts of dry starch powder to get granules for compression containing 1%, 2%, 4%, 8%, 15%, 30%, and 60% of dry starch.

The material for compression containing HCMC was made by adding the HCMC to the sulfathiazole powder, mixing, adding starch paste with some additional water, granulating, and drying. Granule size was standardized with No. 20 and No. 60 mesh sieves also. The preparation of the six HCMC-sulfathiazole granulations is summarized in Table I.

The granulations containing 15% and 30% HCMC become slightly tan during the drying process. This appeared to have been caused either by an unnecessarily protracted drying time or by a slightly excessive drying temperature. These granulations dried very rapidly.

Compressing the Tablets.—Seven sulfathiazole granulations employing dry starch powder as disintegrant and six employing HCMC as the disintegrant were used for compression into tablets. Portions of 0.330 Gm. each were weighed and com-

* Received August 30, 1957, from the laboratories of the University of Wisconsin School of Pharmacy, Madison.
† Present address: Smith Kline and French Laboratories, Philadelphia 1, Pa.

TABLE I.—COMPOSITION OF HCMC-SULFATHIAZOLE GRANULATIONS

Granulation No. →	1	2	3	4	5	6
HCMC, per cent	1	2	4	8	15	30
Sulfathiazole, Gm.	96	95.1	93.1	89.2	82.4	67.9
10% Starch paste, Gm.	30	29	29	28	26	21
HCMC, Gm.	1	2	4	8	15	30
Additional water, cc.	14	14	23	27	36	60
Drying time, hr. 45°	2	2	2	2	2	1.5

pressed into tablets using a special $^3/_8$-inch punch and die set and a mechanical lever machine of a type previously described (6). The seven compressional forces used were 500, 1,000, 1,500, 2,000, 2,500, 3,000, and 4,000 pounds per tablet. The 4,000-lb. level corresponds to a pressure of 18 tons per sq. in. Good tablets are often prepared industrially at pressures of approximately 10 tons per square inch (28). Hence this report deals with a range that is significant in actual tablet manufacturing. Each of the 13 granulations was compressed into tablets at the seven force levels mentioned. Twenty tablets from each of the starch-sulfathiazole granulations and 12 tablets from each of the HCMC-sulfathiazole granulations were punched. Half of each separate group of tablets was used for the disintegration time determinations and half for the hardness tests.

The granulation containing 60% dry starch was not amenable to compression into tablets even under a compressional force of 10,000 lb.

Hardness Test.—Tablet hardness was determined with the Strong-Cobb tablet hardness tester (7, 29).

Disintegration Time Determination.—The method used for determining the disintegration times of the tablets was essentially that of the U. S. P. XIV (30) with the following modifications. The basket rack contained four compartments instead of the six recommended in the U. S. P. Water was used as the disintegration fluid, and its temperature was maintained at 37°. The submerged basket rack was raised and lowered through approximately a 5-cm. range 31 times a minute. A tablet was considered disintegrated when all of its fragments passed through the No. 8 mesh screen floor of its compartment in the basket rack.

RESULTS AND DISCUSSION

As shown in Fig. 1, scale readings of the Strong-Cobb hardness tester are linear with respect to pressure as determined with a compression tank and gauge. The results of the disintegration time and hardness tests are shown graphically in Figs. 2–5. Median values were used in this study. They were used because the number of tablets in each group tested was relatively small so that the occasional widely variant value encountered would affect the mean to an extent not commensurate with its own significance.

The probable errors figured for all the data taken on the basis of mean values were between 2% and 15%. Since median (not mean) values were used for the graphs, the errors should be less than the calculated ones because the occasional widely divergent results are eliminated.

Observation of Figs. 2 and 3 indicates increased disintegration time as the compressional force used

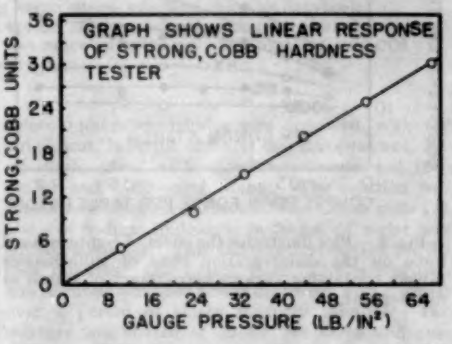

Fig. 1.—Plot shows the result of calibrating the Strong-Cobb hardness tester with a tank of compressed air.

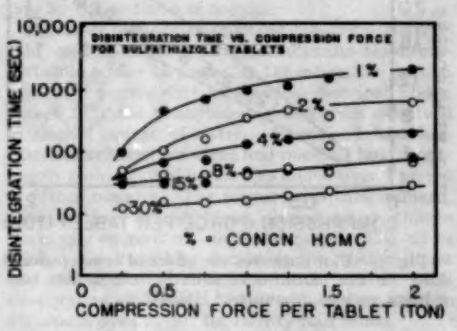

Fig. 2.—Graph shows the effect of compressional force on the disintegration time of sulfathiazole tablets containing various percentages of HCMC as disintegrant.

to make the tablets is increased. It is seen that the time of disintegration decreases greatly as the percentage of the disintegrant is increased. As the compressional forces are increased there is little difference between the disintegration times of the tablets containing 15% and 30% starch or HCMC.

In Figs. 4 and 5 it is observed that hardness generally increases quite rapidly with increase in compressional force. These figures are shown in the form of swath-graphs because the hardness values covered by the swath are primarily a function of the compressional force and appear to be much less dependent on disintegrant concentration. Hence it is not helpful to trace the individual lines which

cross and intermingle frequently. In general, however, tablet hardness appeared to decrease with disintegrant concentration, at least at the higher levels. This is most evident at the 30% starch concentration where tablets were relatively soft at all compressional forces because of the poor compressibility of this component.

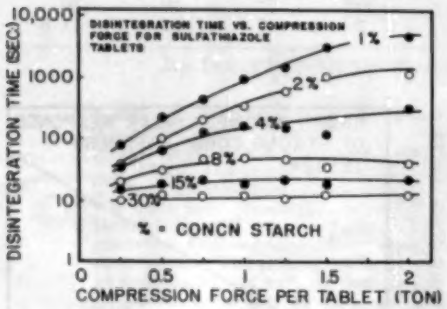

Fig. 3.—Plot illustrates the effect of compressional force on the disintegration time of sulfathiazole tablets containing various percentages of starch as disintegrant.

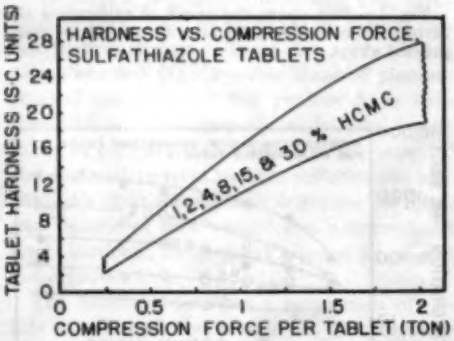

Fig. 4.—Plot indicates the effect of compressional force on the hardness of sulfathiazole tablets containing various amounts of HCMC.

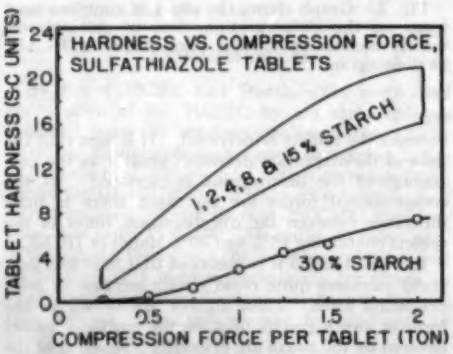

Fig. 5.—Graph demonstrates the effect of compressional force on the hardness of sulfathiazole tablets containing various amounts of starch.

The principal advantages of HCMC in this sulfathiazole formulation are: (*a*) It can be granulated wet with the other tablet ingredients without losing its disintegrant properties upon drying; hence, an extra weighing and mixing step in the tablet manufacturing procedure is eliminated; (*b*) wet granulations employing HCMC dry faster than the same granulations without it; (*c*) since HCMC is granulated along with the other ingredients, the tablets containing HCMC as the disintegrant are somewhat harder than the corresponding tablets containing starch, because no extra "fines" are added to the former. Starch, however, has advantages when the choice of a disintegrant is considered solely on the basis of cost and availability.

A comparison of all the data indicates that there is no great difference between HCMC and starch as regards hardness and disintegration times of sulfathiazole tablets where they are used as disintegrants. These and previously published results (2, 3, 5) are in concordance with the view that numerous polysaccharide acids and synthetic cation exchangers may be expected to possess useful tablet disintegrant properties by virtue of their swelling in water. This study is suggestive of a procedure that may be used for evaluating such or other new materials for utility as tablet disintegrants as they become available.

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Synthesis and Antifungal Studies on New Organic Sulfur Compounds*

By AMRUTLAL M. SHAH and JAMES W. JONES†

Six derivatives of *o*-phenylenediamine-bis-dithiocarbamic acid and six derivatives of 4-chloro-*o*-phenylenediamine-bis-dithiocarbamic acid were prepared. Twelve new compounds, undecylenic acid and ethanol were screened for antimycotic activity against *M. Gypseum*, *M. Canis*, *T. Rubrum*, and *T. Mentagrophyte* by agar plate, paper-disk technique. All the compounds were more effective than undecylenic acid against all of the organisms with exception of the sodium salts of *o*-phenylenediamine-bis-dithiocarbamic acid which was more effective only against *M. Canis* and *T. Rubrum*.

VITAL STATISTICS reveals that fungal diseases of men are growing health problems (1). The search for antifungal agents has been in progress for several decades. Sulfur has long played an important role in the combat between humans and lower form of life as pests, fungi, and bacteria. Horsfall (2) reviewed the antimycotic therapy and presented a classification of antifungal agents. More recently organic sulfur compounds have been considered to be valuable as antifungal agents. Extensive researches have been carried out by plant pathologists for utilizing these organic sulfur compounds, dithiocarbamic acid and its derivatives, as agricultural fungicides (3,4). The above compounds showed remarkable antimicrobial activity for the treatment of fungus diseases of plants, however, very little attention has been given to their use in human chemotherapy.

Several esters of ethylene-bis-dithiocarbamic acid have been prepared in this laboratory and tested against human pathogenic fungi. Some of the compounds were found to be as active as undecylenic acid. The pungent odor and chemically unstable nature of these compounds is a hindrance to their pharmaceutical application (5).

In this investigation, an attempt has been made to prepare several esters of *o*-phenylenediamine-bis-dithiocarbamic acid and 4-chloro-*o*-phenylenediamine-bis-dithiocarbamic acid.

EXPERIMENTAL

Synthesis

***A*.—Disodium *o*-phenylenediamine-bis-dithiocarbamate, *B*.—Disodium 4-chloro-*o*-phenylenediamine-bis-dithiocarbamate.**—One-eighth mole (13.5 Gm.) of *o*-phenylenediamine or 17.64 Gm. of 4-chloro-*o*-phenylenediamine was dissolved, with the aid of heat, in 50 ml. of water in a three-necked, 500 ml. flask, fitted with a reflux, mercury-seal condenser and a dropping funnel. The solution was cooled to 30° and a solution of one-fourth mole (11 Gm.) of sodium hydroxide in 50-ml. of water was added in one lot. This mixture was stirred for fifteen minutes. One-fourth mole (22 Gm.) of carbon disulfide was then added dropwise, with stirring, over a period of thirty to forty minutes. The mixture was stirred at 30–40° for three and one-half hours. The solution was then evaporated to a small volume and an excess of acetone was added. This mixture was placed in a refrigerator until crystallization was complete. The crystals were purified by several recrystallizations from aqueous solution by the addition of acetone.

Esters of *o*-phenylenediamine-bis-dithiocarbamic acid and 4-chloro-*o*-phenylenediamine-bis-dithiocarbamic acid.—In preparing the esters, the sodium salts of the acids were prepared as described above except that the phenylenediamines were dissolved in alcohol instead of water. After the initial mixture had refluxed for three and one-half hours, one-fourth mole of alkyl halide was added over a period of fifteen minutes. The mixture was then refluxed, with stirring, for six hours, during which time a dark, oily or resinous mass separated. The oil or mass was collected by decanting the mother liquor and extracting the residue with ether. After evaporating off the ether, the residues were dried in a vacuum oven at 50° for twenty-four hours.

The yields and N analyses are shown in Table I.

Mycological

The compounds synthesized were tested for antifungal activity by the procedure described by Klingman and Rosenweig (6) for the determination of fungicidal activity *in vitro*.

Table I lists the structural formulas and numbers of the compounds tested for antimycotic activity. The results of the mycological work are summarized in Tables II, III, IV, and V.

DISCUSSION

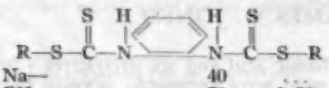
It was found that the sodium salts and the methyl and ethyl esters of the dithiocarbamic acids were unstable and decomposed at room temperature while the allyl, epoxypropyl, and benzyl esters were stable. According to Horsfall, *et al.* (7), decomposition may be due to formation of the corresponding isothiocyanates.

* Received June 27, 1957, from the College of Pharmacy, State University of Iowa, Iowa City.

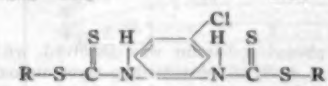
Abstracted from a dissertation submitted to the Graduate College of the State University of Iowa by Amrutlal M. Shah in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Professor of Pharmacy, College of Pharmacy, State University of Iowa, Iowa City.

TABLE I.—COMPOUNDS SYNTHESIZED AND/OR TESTED



	R	Yield, %	N Calcd., %	N Found, %
1.	Na—	40	...	...
2.	CH_3—	70	9.79	9.58
3.	C_2H_5—	40	8.86	8.92
4.	CH_2=$CHCH_2$—	50	8.21	8.15
5.	CH_2—CH—CH_2— (O)	47	7.50	7.32
6.	C_6H_5—CH_2—	37	6.34	6.41



	R	Yield, %	N Calcd., %	N Found, %
7.	Na—	39	...	...
8.	CH_3—	69	8.66	8.50
9.	C_2H_5—	76	7.98	7.89
10.	CH_2=CH—CH_2—	55	7.46	7.31
11.	CH_2—CH—CH_2— (O)	80	6.86	6.90
12.	C_6H_5—CH_2—	80	5.89	5.71
13.	Undecylenic Acid	...	...	...
14.	Ethanol	...	...	...

The procedure used for testing antifungal activity does not give the inhibition value as an end point, which is the minimum concentration required to inhibit the growth of a certain species. The method gives a preliminary indication of the potential which a compound might possess in comparison to a standard antifungal agent which is used frequently, undecylenic acid.

The Tables II, III, IV, and V indicate that most of the compounds were active against one or more strains of fungi. One factor which influences the width of inhibition zone is the speed with which the organism grows. *M. Gypseum* and *T. Mentagrophyte* seem to grow considerably faster than *M. Canis* and *T. Rubrum*.

Compounds 1 and 7 are water-soluble sodium salts. Generally, they were less active than undecylenic acid against all 4 organisms. Compounds 2 and 8, the methyl esters, showed more activity than undecylenic acid against all 4 organisms. Klopping and Van der Kerk (8) showed that optimum activity occurs with the lower alkyl esters, i.e., the methyl and ethyl esters of dithiocarbamic acids. The results in this work agree with that observation. Compounds 3 and 9, the ethyl esters, were considerably more active than undecylenic acid against all 4 organisms, less active than compounds 2 and 8 against *M. Gypseum* and *T. Mentagrophyte* and about equally as active against *M. Canis* and *T. Rubrum*.

TABLE II.—ZONES OF INHIBITION IN MILLIMETERS AGAINST M. GYPSEUM

Compound No.	1%	2%	3%	4%	5%
1	0	0	0	0.25	0.25
2	0.25	1.00	3.25	4.25	7.25
3	0	0.25	1.00	2.25	4.00
4	0	1.25	4.00	4.50	6.50
5	0	0	5.00	1.00	1.50
6	2.00	3.50	7.00	8.75	10.50
7	0	0	0	0	0
8	5.25	10.25	11.50	15.00	15.50
9	4.50	9.00	10.75	12.00	13.00
10	1.50	3.00	6.00	7.50	9.75
11	1.25	1.75	2.75	3.25	5.25
12	5.75	9.00	9.75	10.50	12.00
13	0	0	0.25	0.50	1.50
14	0	0	0	0	0

TABLE III.—ZONES OF INHIBITION IN MILLIMETERS AGAINST T. RUBRUM

Compound No.	1%	2%	3%	4%	5%
1	9.75	13.00	15.75	19.00	20.50
2	11.75	12.75	18.25	19.50	21.00
3	11.50	14.50	18.25	21.25	21.50
4	13.00	17.50	18.00	18.75	20.25
5	4.50	11.50	13.25	16.75	18.75
6	13.25	14.00	15.50	16.25	18.50
7	3.50	5.00	6.00	7.00	8.75
8	13.25	15.75	16.00	17.00	18.25
9	10.00	11.50	12.00	13.25	17.75
10	11.50	12.25	12.50	14.00	18.50
11	10.25	12.25	13.25	15.00	15.50
12	14.00	15.00	18.25	20.75	20.75
13	6.25	8.00	8.50	9.25	9.75
14	0	0	0	0	0

TABLE IV.—ZONES OF INHIBITION IN MILLIMETERS AGAINST M. CANIS

Compound No.	1%	2%	3%	4%	5%
1	9.50	11.00	13.00	14.25	17.50
2	10.00	13.00	15.50	16.00	21.00
3	9.75	11.75	15.25	17.00	18.00
4	8.00	11.50	12.50	13.00	14.25
5	3.00	4.25	6.25	8.25	9.50
6	9.50	10.75	11.00	13.50	14.50
7	0	2.00	3.00	3.25	4.00
8	7.50	12.75	13.50	16.75	18.25
9	8.75	13.50	15.25	17.00	18.00
10	8.00	9.50	13.50	15.00	15.25
11	9.00	9.50	10.00	10.50	11.50
12	12.25	13.25	14.25	16.00	17.75
13	2.25	3.25	5.50	7.25	7.50
14	0	0	0	0	0

TABLE V.—ZONES OF INHIBITION IN MILLIMETERS AGAINST T. MENTAGROPHYTE

Compound No.	1%	2%	3%	4%	5%
1	0	0	0	0	2.00
2	5.75	7.25	9.50	9.75	11.00
3	2.00	3.00	5.50	6.00	6.50
4	1.00	2.00	2.50	5.25	6.50
5	2.00	2.00	2.50	4.00	4.50
6	2.75	6.00	7.75	8.00	9.25
7	0	0	0	2.00	2.25
8	4.75	6.00	7.75	9.25	11.25
9	2.75	4.50	6.00	8.00	8.50
10	4.00	5.75	7.25	9.00	10.00
11	2.00	2.50	3.00	4.25	5.00
12	6.75	7.25	8.25	9.25	10.75
13	2.00	2.75	3.00	3.50	4.00
14	0	0	0	0	0

The decrease in activity might be due to the ester chain being increased with a consequent decrease in the extent of ionization. Compounds 4 and 10, the allyl esters, were considerably more active than undecylenic acid against all 4 organisms, but showed an activity which, in general, was less than that of the methyl esters. Again, a decrease in antifungal activity by lengthening the carbon chain in the ester part of molecule was shown. However, the allyl esters showed more activity than the epoxypropyl esters against all 4 organisms. According to Friedman (9), this greater activity may be due to the —CH=CH— group. Compounds 6 and 12, the benzyl esters, were more inhibitory against all 4 species than the allyl or epoxypropyl esters but less inhibitory than the methyl esters with some exceptions. The greater activity over the allyl or epoxypropyl ester may be due to the fact that these compounds have a smaller number of aliphatic carbon atoms in the ester chain of molecule. The lesser activity of the benzyl esters than that shown by the methyl esters may be due to the higher molecular weight of the ester group, which may decrease the tendency of the compounds to ionize causing them to be less effective.

Compound 14, ethanol, showed no inhibition against any of the fungi used. It was used as a control since it was used to dissolve the esters.

In the past, many efforts have been made to change the molecular structure of this type of compounds with regard to the thiocarbamate portion and to compare their activities. The results of this investigation suggest that dithiocarbamic acid derivatives prepared from *o*-phenylenediamine and 4-chloro-*o*-phenylenediamine possess a higher antifungal activity than derivatives prepared from ethylenediamine.

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The Pharmacology of 4-[2-(5-Phenyloxazoyl)]-1-Methylpyridinium Chloride and *p*-Toluenesulfonate*

By THOMAS J. HALEY, ANNA M. FLESHER, and N. KOMESU

It has been shown that 4-[2-(5-phenyloxazoyl)]-1-methylpyridinium chloride or *p*-toluenesulfonate produced a dose dependent hypotension which could not be modified by atropine and pyrilamine maleate. There was no significant effect on respiration until exitus. Electrocardiographic changes indicate that these compounds are toxic to the heart. There was no evidence that the pyridinium compounds had any cholinergic, adrenergic or ganglionic blocking properties. Although the pyridinium compounds produced hypothermia, their potency was of a low order, while their toxicity was of a high order, thus preventing use in therapeutics. The pyridinium compounds do not induce or prevent chromoachryodynia in rats, but their vasodilator effect does prevent epinephrine death in mice.

RECENTLY, Lushbaugh, *et al.* (1), reported that the oxazole quaternary salts produced poikilothermia in mice. Haley, *et al.* (2), observed that these compounds also produced hypotension, but had no effect on autonomic ganglia. Ott, *et al.* (3), synthesized the chloride and *p*-toluenesulfonate salts of a new compound, 4-[2-(5-phenyloxazoyl)]-1-methylpyridine. The quaternary nitrogen is in the pyridyl rather than the oxazoyl ring (Fig. 1). We have investigated these compounds to determine what differences

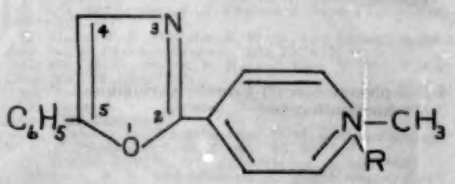

Fig. 1.—Substituted quaternary pyridinium compounds: R = Cl or $CH_3C_6H_4SO_3$

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in pharmacological activity have been produced by such structural changes.

EXPERIMENTAL

Nineteen cats of both sexes, weighing 2.2 to 3.85 Kg., were anesthetized with 35 mg./Kg. of sodium pentobarbital intraperitoneally. Blood pressure was recorded from the carotid artery with a mercury manometer. Respiration was recorded by the method of Haley (4). *In situ* intestinal contractions were recorded with a balloon-tambour system. The ECG was obtained with Lead II of a Sanborn Viso-Cardiette. Ganglionic effects were studied by recording contractions of the nictitating membrane following stimulation of the preganglionic cervical sympathetic fibers with 8V/10 sec. from a Grass Model S-4 Stimulator. Similar stimulation of the vagus nerve was used to study anticholinergic activity. Five spinal cats were prepared in the usual manner. Doses of the drugs used were: pyridinium compounds, 0.5–15 mg./Kg., epinephrine, 3 mcg./Kg., acetylcholine, 5 mcg./Kg.; histamine, 0.1 mcg./Kg., atropine, 2 mg./Kg.; and pyrilamine maleate, 1 mg./Kg. The blocking drugs were given one-half hour prior to the pyridinium compounds and tests were made after each dose to establish the maintenance of the blockade throughout the experiment. Effects on body temperature were studied in CF-1 mice using electrical thermometers according to the method of Clark and Trolander (5). Cholinergic stimulant effects were studied using the chromoachryodynia response of the rat (5, 7). Effects on epinephrine mortality in mice were studied by the method of Loew and Micetich (8). Ocular effects were studied after topical application to the eyes of New Zealand rabbits.

RESULTS

Cardiovascular and Respiratory Effects.—Both pyridinium compounds produced a hypotension which depended upon the initial blood pressure and the dose administered, see Table I. A response plateau usually developed at the 10 mg./Kg. dose level. With most animals, there was an abrupt hypotensive response followed by a progressive continuous decrease in mean arterial pressure. The duration of effect varied with each dose and usually was of the order of fifteen to twenty minutes at the 5 mg./Kg. dose. The pyridinium compounds appeared to have some cumulative action, possible on receptors in the peripheral vascular system, because there was a tendency to bleed from the incisions associated with the insertion of the various recording devices. This effect became pronounced after the 5 mg./Kg. dose at which time the animals had received a total dose of 8.5 mg./Kg. Based on an equivalent hypotension, acetylcholine is 1,000 times and histamine 5,000 times more active than the pyridinium compounds. Neither atropine nor pyrilamine maleate was able to block the pyridinium compound induced hypotension, although the former blocked the acetylcholine response and the latter blocked the histamine response. However, they did reduce the progressive decrease in blood pressure which followed the initial abrupt fall. This stabilization of the blood pressure resulted in greater responses at the higher doses. In the spinal cat, both pyridinium compounds produced their usual hypotensive response.

Neither pyridinium compound had any great effect on respiration except to produce a slight transient increase in depth coincident with the hypotension. Cheyne-Stokes respiration occurred sporadically in several animals. Death was due to respiratory failure coupled with cardiovascular collapse.

Effects on the Electrocardiogram.—Both pyridinium compounds had a progressive negative chronotropic effect on the cat heart *in situ*, finally reducing the heart rate 40 to 60 beats/minute. This effect was not modified by atropine and pyrilamine maleate. There was a transient increase in the height of the T-wave with each dose of pyridinium compounds, indicative of cardiac anoxia. Of a more serious nature was the observation of auricular fibrillation, sinus arrhythmia, trigeminy, ventricular tachycardia, left bundle branch block and left ventricle strain. These electrocardiographic changes were observed in the majority of animals and were only partially blocked by atropine and pyrilamine maleate. At exitus all cats showed auriculoventricular dissociation, but this occurred only after respiratory paralysis.

Effects on the Autonomic Nervous System.—Neither compound had any effect on the response

TABLE I.—HYPOTENSIVE EFFECTS OF QUATERNARY PYRIDINIUM COMPOUNDS

Compound	Dose, mg./Kg.	Hypotension in Millimeters of Mercury	
		No. Blocking Drug, Mean ± S. E.	Atropine and Pyrilamine Maleate, Mean ± S. E.
4-2-(5-phenyloxazoyl)-1-methylpyridinium chloride	0.5	47 ± 1.2	26 ± 3.8
	1.0	51 ± 3.1	43 ± 2.4
	2.0	74 ± 4.1	71 ± 4.1
	5.0	82 ± 3.6	103 ± 7.2
	10.0	71 ± 6.4	100 ± 6.5
	15.0		63 ± 9.0
4-2-(5-phenyloxazoyl)-1-methylpyridinium-*p*-toluenesulfonate	0.5	41 ± 4.04	27 ± 1.0
	1.0	42 ± 4.09	35 ± 2.7
	2.0	38 ± 2.95	44 ± 6.6
	5.0	77 ± 10.28	88 ± 5.5
	10.0	81.5 ± 11.3	88 ± 7.5
	15.5	76 ± 13.9	91 ± 9.3

of the nictitating membrane to preganglionic stimulation; also they did not block the response produced by intravenous epinephrine or acetylcholine. The pyridinium compounds did not cause contraction of the nictitating membrane at any of the doses used. No adrenergic blocking or atropine-like effects of the pyridinium compounds were observed when epinephrine or acetylcholine was administered intravenously. Furthermore, the effects of vagal stimulation on the heart, blood pressure, and intestinal motility were not inhibited by the pyridinium compounds.

Effects on Intestinal Motility *in Situ*.—Both compounds increased the tonus, amplitude, and rate of contraction of the cat intestine at all doses used and the maximum effect was obtained at 5 mg./Kg. Such effects were greater than those produced by acetylcholine and had a longer duration. The intestinal relaxation produced by epinephrine was not affected by the pyridinium compounds.

Effects on Body Temperature.—Administration of 100 mg./Kg. of the methochloride derivative reduced mouse body temperature from the control value of 30° to 25.5° within three hours, but four of five mice died. With the methyl-*p*-toluenesulfonate derivative, a dose of 200 mg./Kg. killed six of six mice over a period of five hours, thus preventing any evaluation of hypothermic effect. A dose of 150 mg./Kg. reduced body temperature from the control 30° to 26.2° six hours after injection, at which time three of six animals died. The 100 mg./Kg. dose was not lethal but reduced body temperature only 0.5°.

Miscellaneous Effects.—After topical application of 1% solutions of the pyridinium compounds to the eyes of rabbits, there was no evidence of irritation, local anesthesia or changes in pupillary size.

Prior intraperitoneal administration of the pyridinium compounds prevented death from intravenous epinephrine under the conditions specified by Loew and Micetich (8), see Table II. This protectant action was probably related to the vasodilation produced by the pyridinium compounds because these compounds are devoid of adrenergic blocking activity. In this regard the results were similar to those observed with acetyl-β-methylcholine (8). All of the mice receiving the pyridinium compounds were greatly depressed, quiescent, and cold to the touch. The compounds also appear to have a low renal threshold because they rapidly appeared in the urine giving it their characteristic fluorescent yellow color. It was quite evident that the methochloride derivative was more toxic than the methyl-*p*-toluenesulfonate derivative and that both compounds become more toxic after the epinephrine injection, see Table II.

A profuse chromoachryodynia was produced in rats by 10 mg./Kg. of acetyl-β-methylcholine but neither pyridinium compound was capable of either initiating or blocking this effect at doses of 100, 250, or 500 mg./Kg. Furthermore, the two higher doses were lethal within five to ten minutes and the smaller dose shortly after the test dose of acetyl-β-methylcholine.

DISCUSSION

The substitution of the pyridyl group for the 1-naphthyl, 4-methylphenyl or 4-methoxyphenyl group in position 2 of the oxazole ring with the quaternary nitrogen in the pyridyl group, rather than in the oxazoyl group, increases the overall toxicity and decreases the hypothermic activity of the compound. This also introduces a series of detrimental myocardial effects which would prevent any application of the pyridinium compounds in human therapeutics. In all other aspects, both series of compounds appear to produce almost equivalent pharmacological effects. In so far as their hypotensive actions are concerned, the data indicate that the pyridinium compounds produce most of this effect by peripheral vasodilation although a partial cardiac component cannot be entirely ruled out.

TABLE II.—EPINEPHRINE TOXICITY ANTAGONISM BY PYRIDINIUM COMPOUNDS

Compound	I. P. Dose, mg./Kg.	Total Mortality by Hours 1	5	7	24
Saline 0.9%	1 ml.	7/10	8/10	8/10	9/10
4-2-(5-phenyloxazoyl)-1-methylpyridinium chloride	75	0/10[a]	0/10	1/10	1/10
		0/10	2/10	5/10	10/10
	100	1/10[a]	4/10	4/10	5/10
		0/10	6/10	10/10	10/10
4-2-(5-phenyloxazoyl)-1-methylpyridinium-*p*-toluenesulfonate	75	0/10[a]	0/10	0/10	0/10
		0/10	0/10	2/10	8/10
	100	0/10[a]	0/10	0/10	1/10
		0/10	1/10	5/10	10/10

[a] Control no epinephrine, all other mice received 14 mg./Kg. of epinephrine hydrochloride intravenously thirty minutes after the pyridinium compound.

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Chemical Composition of Gum Turpentines of Pines XXIX.*

A Report on *Pinus ponderosa* from Five Localities: Central Idaho, Central Montana, Southeastern Wyoming, Northwestern Nebraska, and Central Eastern Colorado

By N. T. MIROV and P. M. ILOFF, Jr.

Five samples of *Pinus ponderosa* turpentine were analyzed. The sample from southwestern Idaho was composed of: *l*-α-pinene, 6 per cent; *l*-β-pinene, 30 per cent; β-myrcene, 3 per cent; *d*-Δ^3-carene, 40 per cent; *l,dl*-limonene, 10 per cent; terpinolene, 2 per cent; and cadienene, 5 per cent. The central Montana sample contained: *l*-α-pinene, 3 per cent; *l*-β-pinene, 25 per cent; β-myrcene, 5 per cent; *d*-Δ^3-carene, 47 per cent; *l,dl*-limonene, 6 per cent; terpinolene, 4 per cent; longifolene, 2 per cent; and cadinene, 5 per cent. The sample from southeastern Wyoming was composed of *l*-α-pinene, 2 per cent; *l*-β-pinene, 31 per cent; β-myrcene, 4 per cent; *d*-Δ^3-carene, 40 per cent; *l,dl*-limonene, 4 per cent; terpinolene, 1 per cent; longifolene, 10 per cent; and cadinene, 5 per cent. Southwestern Nebraska turpentine contained: *l,dl*-α-pinene, 2 per cent; *l*-β-pinene, 32 per cent; β-myrcene, 3 per cent; *d*-Δ^3-carene, 38 per cent; *l,dl*-limonene, 7 per cent; longifolene, 12 per cent; and cadinene, 4 per cent. The sample from southeastern Colorado was composed of: *dl*-α-pinene, 5 per cent; *l*-β-pinene, 17 per cent; β-myrcene, 10 per cent; *d*-Δ^3-carene, 40 per cent; *l,dl*-limonene, 15 per cent; and longifolene, 8 per cent.

THE CHEMICAL COMPOSITION of turpentine of *Pinus ponderosa* from several localities has been reported in previous articles of the present series. These localities were: Sierra Nevada of California, coastal mountains of California, northern Idaho, Utah, Arizona, Black Hills of South Dakota, and Rocky Mountains of central Colorado (2–5). They represent only part of the tree's natural range. *P. ponderosa* occurs from the Pacific Coast to Nebraska and from the southern part of western Canada to the Mexican border. Therefore it appeared desirable to analyze turpentines of this pine from some additional, "strategically" situated places.

This paper reports the composition of turpentine obtained from five other localities; southwestern Idaho, central Montana, southeastern Wyoming, northwestern Nebraska, and southeastern Colorado.

EXPERIMENTAL

Turpentine was obtained by heating, under reduced pressure, the oleoresin samples from these five localities. At the end of each operation, when all the turpentine was recovered, the temperature of the pot residue (rosin) was increased to 200° and the pressure was reduced to 0.1 mm.

Pinus ponderosa from Southwestern Idaho.—A sample of oleoresin was collected near Idaho City, Idaho, at an elevation of 4,070 feet.

The turpentine amounted to 26.5% of the weight of oleoresin and had the following physical characteristics: d_4^{23}, 0.8563; n_D^{23}, 1.4758; $[\alpha]_D^{23} - 7.2$. A 404-Gm. batch of turpentine was distilled at a pressure of 11 mm. in a Todd column, 0.25 mm. inside diameter, packed with $^3/_{32}$-inch glass helices, and equipped with a Todd automatic reflux head. A reflux ratio of 10:1 was maintained.

We had learned previously that the bulk of *P. ponderosa* turpentine from any locality consists predominantly of Δ^3-carene and varying amounts of α- and β-pinenes. Accordingly, the percentage of α-pinene, β-pinene and Δ^3-carene were determined by physical properties alone, using Sutherland's method (7).

We estimated that the turpentine sample of *Pinus ponderosa* from southwestern Idaho contained 6% of *l*-α-pinene (fractions 1 to 5), 30% of *l*-β-pinene (fractions 6 to 10), and 40% *d*-Δ^3-carene (fractions 11 to 14).

A slight change in physical properties of Fraction 12 indicated the possible presence of a substance other than Δ^3-carene. This fraction was analyzed for β-myrcene using the method of Berry (1). Ten grams of the oil and 0.8 Gm. of maleic anhydride were heated for thirty minutes at 100°. The solution was cooled, 20 ml. of hexane was added, and the resulting mixture was kept at −5° for two days. The resulting crystals were recrystallized from hexane twice, and 0.44 Gm. of crystals were received. The crystals possessed a melting point of 33.5 to 34.5°. The melting point was not depressed by admixture of maleic anhydride adduct of known β-myrcene.

The physical properties of fraction 19 indicated a probable presence of *l,dl*-limonene. A 2-ml. sample of the fraction was treated with bromine according

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to the method of Berry (1). After one month of refrigeration, the reaction mixture was filtered and 50 mg. of crude tetrabromide was received. The tetrabromide was recrystallized twice from ethyl acetate, after which it melted at 124–125°. The melting point was not depressed when an authentic *dl*-limonene tetrabromide was admixed.

Fraction 24 had physical properties which indicated the presence of terpinolene. Two grams of the fraction were treated with bromine using the procedure of Berry (1); 2.60 Gm. of crude tetrabromide were obtained. After six recrystallizations from ethyl acetate a tetrabromide, m. p. 116–117°, was received. The melting point was not depressed by admixture with authentic terpinolene tetrabromide.

Fraction 28 consisted mainly of sesquiterpenes and had the following properties: d_4^{24}, 0.9159; n_D^{24}, 1.5031; $[\alpha]_D^{24}$ − 33.4. This fraction was redistilled over sodium at 14 mm. pressure; all fractions had negative rotations. A heart cut had the following properties: b_{14} 130–135°, d_4^{26}, 0.9104; n_D^{25}, 1.5036; $[\alpha]_D^{26}$ − 30.1.

A 3-ml. portion of the heart cut was used to prepare 200 mg. of a hydrochloride which after three recrystallizations from glacial acetic acid had a melting point of 120–120.5°. Admixture of *l*-cadinene hydrochloride did not depress the melting point.

To sum up, *P. ponderosa* turpentine from southwestern Idaho was composed of *l*-α-pinene, 6%; *l*-β-pinene, 30%; *d*-Δ³-carene, 40%; β-myrcene, 3%; *l,dl*-limonene, 10%; terpinolene, 2%; and cadinene, 5%.

Pinus ponderosa from Central Montana.—The Montana sample of turpentine was distilled from oleoresin collected near the town of White Sulphur Springs, along the course of Whitetail Deer Creek, at an elevation of 5,000 feet.

The yield of turpentine was 17.7%; its physical characteristics were these: d_4^{23}, 0.8614; n_D^{23}, 1.4760; $[\alpha]_D^{23}$ − 0.9. A 330-Gm. batch was distilled through the Todd column, described above, at 10 mm. pressure. A reflux ratio of 10:1 was maintained.

Sutherland's (7) density/index of refraction diagram indicated that fractions 1 and 2 consisted mainly of *l*-α-pinene (about 3% of the total). Fractions 3 to 10 were those of *l*-β-pinene (25%). Fractions 11 to 14 contained *d*-Δ³-carene (47%). Fraction 11 contained about 5% of β-myrcene. This open-chain terpene was identified by preparing a maleic anhydride adduct, according to Berry (1). Admixture of known maleic anhydride adduct of myrcene did not depress the melting point of the substance.

A rapid increase of *levo*-rotation of the following fractions, peaking in fraction 19, caused us to suppose that fractions 15 to 21 contained *l,dl*-limonene.

Two milliliters of fraction 19 were treated with bromine by the method of Berry (1); 100 mg. of crude tetrabromide were received. After three recrystallizations from ethyl acetate the tetrabromide melted at 124.5° and was not depressed in melting point by admixture of authentic *dl*-limonene tetrabromide.

Fraction 22 had properties indicating the presence of terpinolene. Two grams of the fraction were treated with bromine using the procedure of Berry (1); 2.75 Gm. of crude tetrabromide were obtained. After four recrystallizations from ethyl acetate, 0.31 Gm. of tetrabromide, m. p. 116–117°, was received. The melting point was not depressed by admixture of authentic terpinolene tetrabromide. About 8% of the charge boiled above 105° at 10 mm.; it consisted mainly of sesquiterpenes. The properties of the sesquiterpene fractions indicated that there were apparently two sesquiterpenes, which occurred in the highest purity in fraction 28 and fraction 29.

Fraction 28 had the following physical properties: d_4^{23}, 0.9321; n_D^{23}, 1.5120; $[\alpha]_D^{24}$ +36.3.

A 3-ml. portion of fraction 28 was used to prepare a hydrochloride. After two recrystallizations from glacial acetic acid, 0.79 Gm. of crystals were received; the crystals melted at 58.5–59°. Admixture of known hydrochloride of *d*-longifolene showed no depression.

Fraction 29 had the following physical properties: d_4^{23}, 0.9159; n_D^{23}, 1.5052; $[\alpha]_D^{24}$ −28.2. A 3-ml. portion of fraction 29 was used to prepare 0.33 Gm. of hydrochloride. After two recrystallizations from glacial acetic acid, 72 mg. of hydrochloride (m. p. 120–121°; α_D^{23} −41, c = 1.32 in chloroform) were received. Admixture of known *levo*-rotatory cadinene hydrochloride did not depress the melting point.

To sum up, turpentine obtained from *Pinus ponderosa* growing in central Montana contained: *l,dl*-α-pinene, 3%; *l*-β-pinene, 25%; β-myrcene, 5%; *d*-Δ³-carene, 47%; *l-dl*-limonene, 6%; terpinolene, 4%; longifolene, 2%; cadinene, 5%.

Pinus ponderosa from Southeastern Wyoming.—The material for our work was obtained in the Laramie Mountains near a place called Esterbrook, at an elevation of 6,500 feet. The yield of turpentine was 22.0% of the weight of the oleoresin.

The turpentine had the following characteristics: d_4^{24}, 0.8673; n_D^{25}, 1.4768; $[\alpha]_D^{24}$ +1.5.

A 416-Gm. batch was distilled through the previously mentioned Todd column at 10 mm. pressure. A reflux ratio of 10:1 was maintained.

Composition of fractions 1 to 14 was determined by physical characteristics, according to the method proposed by Sutherland (7), except for the identification of β-myrcene.

Fraction 1 consisted chiefly of *l*-α-pinene (2%); fractions 2 to 8 contained *l*-β-pinene (31 per cent). Fractions 9 to 14 were *d*-Δ³-carene fractions (40% of the total oil). Fraction 10 was analyzed for β-myrcene.

Using Berry's procedure (1), 0.40 Gm. of maleic anhydride adduct, m. p. 33.5 to 34.5°, was produced. The melting point of the adduct was not depressed by admixture of the known maleic anhydride adduct of myrcene. Myrcene amounted to about 4% of the total oil.

The physical properties of fraction 17 indicated the probable presence of *l,dl*-limonene. A 1.5-ml. sample of the oil was dissolved in 1:1 ethanol-ethyl ether and treated at 0° with bromine according to the method of Wallach (8). The solvent was evaporated, and the oil was mixed with methanol. After several days of standing at −5°, the crystals were filtered out and recrystallized three times from ethyl acetate. Sixty milligrams of tetrabromide, m. p. 124–125°, were received. A mixed melting with authentic *dl*-limonene tetrabromide showed no depression. Limonene amounted to 4% of the total oil.

TABLE I.—FRACTIONAL DISTILLATION OF PINUS PONDEROSA TURPENTINE FROM FIVE DIFFERENT LOCALITIES

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
		Pinus ponderosa from Idaho City, Idaho (404 Gm. used)		d_4^{23}	$n_D^{23.5}$	$[\alpha]_D^{23.5}$
1	11	40–42	1.9	0.8540	1.4647	−21.0
2	11	42–44	1.0	0.8547	1.4661	−21.1
3	11	44–45	1.2	0.8573	1.4680	−22.2
4	11	45–46	1.0	0.8573	1.4701	−22.2
5	11	46–47	0.8	0.8588	1.4710	−23.0
6	11	47–49	3.9	0.8573	1.4727	−18.5
7	11	49–51	4.0	0.8521	1.4717	− 8.2
8	11	51	5.1	0.8525	1.4712	− 0.9
9	11	51–52	6.9	0.8536	1.4714	− 2.3
10	11	52–53	9.8	0.8510	1.4718	− 0.4
11	11	53	12.2	0.8499	1.4711	+ 5.9
12	11	53–54	8.4	0.8485	1.4706	+11.3
13	11	54	10.5	0.8503	1.4707	+13.4
14	11	54–55	10.0	0.8536	1.4708	+11.4
15	11	55–56	4.2	0.8544	1.4712	− 4.4
16	11	56–57	1.1	0.8507	1.4720	−34.8
17	11	57–58	1.1	0.8444	0.4726	−55.0
18	11	58–59	0.7	0.8433	1.4729	−71.4
19	11	59–60	1.8	0.8426	1.4732	−84.7
20	11	60–63	0.9	0.8426	1.4749	−78.7
21	11	63–66	0.7	0.8470	1.4791	−44.4
22	11	66–67	0.5	0.8536	1.4823	−20.1
23	11	67–68	1.1	0.8562	1.4847	− 6.8
24	11	68–69	1.2	0.8559	1.4847	− 1.2
25	11	69–82	0.7	0.8596	1.4807	− 4.0
26	11	82–92	0.8	0.9208	1.4959	− 7.2
27	11	92–103	1.1	0.9513	1.5100	0.0
28	11	Above 103	5.2	0.9159	1.5031	−33.4
Residue and losses			3.2			
		Pinus ponderosa from White Sulfur Springs, Montana (330 Gm. used)		d_4^{23}	n_D^{23}	$[\alpha]_D^{23.5}$
1	10	39–41	1.6	0.8577	1.4669	−16.9
2	10	41–42	1.6	0.8595	1.4690	−18.9
3	10	42–43	2.3	0.8610	1.4720	−21.2
4	10	43–44	4.0	0.8625	1.4719	−21.8
5	10	44–45	1.2	0.8632	1.4746	−23.8
6	10	45–46	2.5	0.8632	1.4760	−23.3
7	10	46–47	3.0	0.8625	1.4762	−21.3
8	10	47–48	3.8	0.8606	1.4756	−15.3
9	10	48–49	2.7	0.8566	1.4744	− 8.2
10	10	49–50	4.6	0.8544	1.4738	− 2.1
11	10	50–51	12.0	0.8518	1.4728	+ 5.2
12	10	51–51.5	13.0	0.8521	1.4720	+10.2
13	10	51.5–52	13.0	0.8540	1.4718	+14.1
14	10	52–53	11.5	0.8569	1.4720	+11.6
15	10	53–54	1.0	0.8540	1.4730	− 7.2
16	10	54–55	0.7	0.8507	1.4738	−32.9
17	10	55–56	0.8	0.8485	1.4741	−36.9
18	10	56–58	0.7	0.8448	1.4751	−50.8
19	10	58–60	0.5	0.8448	1.4756	−56.1
20	10	60–64	0.7	0.8477	1.4798	−40.7
21	10	64–66	0.8	0.8532	1.4841	−11.3
22	10	66–67	2.1	0.8547	1.4850	−16.4
23	10	67–74	4.2	0.8628	1.4770	− 8.1
24	10	74–90	1.5	0.9185	1.4878	− 8.4
25	10	90–105	0.3	0.9381	1.4898	− 3.4
26	10	105–110	0.6	0.9392	1.4840	+ 4.0
27	10	110–115	1.8	0.9358	1.4940	+25.4
28	10	115–116	1.3	0.9321	1.5120	+36.3
29	10	Above 116	3.7	0.9159	1.5052	−28.2
Residue and loss			2.5			
		Pinus ponderosa from Esterbrook, Wyo. (416 Gm. used)		d_4^{23}	$n_D^{23.5}$	$[\alpha]_D^{23.5}$
1	10	42–44	2.0	0.8580	1.4670	−12.1
2	10	44–46	1.3	0.8654	1.4700	−16.2
3	10	46–47	2.1	0.8591	1.4722	−18.6
4	10	47–48	6.2	0.8606	1.4737	−18.8
5	10	48–49	4.5	0.8588	1.4738	−15.8

TABLE I.—(*Continued*)

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
6	10	49–50	4.2	0.8580	1.4735	−11.4
7	10	50–51	6.0	0.8555	1.4730	− 6.4
8	10	51–52	6.5	0.8544	1.4723	− 0.9
9	10	52–53	9.7	0.8521	1.4718	+ 4.7
10	10	53	8.0	0.8525	1.4711	+10.2
11	10	53	10.2	0.8533	1.4709	+13.3
12	10	53–54	1.4	0.8577	1.4708	+15.3
13	10	54	9.7	0.8555	1.4709	+15.2
14	10	54–55	3.0	0.8558	1.4716	+10.1
15	10	55–56	1.0	0.8566	1.4728	− 1.0
16	10	56–57	0.6	0.8525	1.4740	−14.1
17	10	57–59	0.4	0.8492	1.4751	−24.5
18	10	59–62	0.4	0.8470	1.4769	−25.4
19	10	62–65	0.5	0.8485	1.4797	−22.1
20	10	65–67	0.5	0.8529	1.4821	−13.6
21	10	67–68	1.2	0.8548	1.4845	− 3.2
22	10	68–70	1.2	0.8548	1.4831	− 0.3
23	10	70–80	0.3	0.8622	1.4784	− 3.4
24	10	80–92	0.6	0.9086	1.4861	− 7.8
25	10	92–105	1.0	0.9433	1.4918	− 3.6
26	10	105–112	1.0	0.9418	1.4858	+ 4.2
27	10	112–115	3.6	0.9381	1.4901	+20.5
28	10	115–117	1.6	0.9344	1.4978	+35.8
29	10	117–118	3.5	0.9326	1.5019	+40.7
30	10	118–119	1.2	0.9311	1.5027	+35.4
31			4.1	0.9134	1.5052	−40.5
Pot residue and loss			2.5			

Pinus ponderosa from Chadron, Nebraska (365 Gm. used)

				d_4^{25}	$n_D^{25.5}$	$[\alpha]_D^{25}$
1	11	42–44	1.8	0.8551	1.4660	− 2.4
2	11	44–46	1.7	0.8548	1.4681	− 7.2
3	11	46–47	1.9	0.8557	1.4693	− 9.3
4	11	47–48	3.2	0.8563	1.4709	−11.7
5	11	48–49	5.0	0.8557	1.4720	−12.2
6	11	49–50	6.0	0.8560	1.4728	−11.8
7	11	50–51	4.2	0.8541	1.4726	− 8.8
8	11	51–52	6.5	0.8519	1.4725	− 6.0
9	11	52–53	5.2	0.8510	1.4720	− 2.8
10	11	53–54	9.9	0.8494	1.4712	+ 4.2
11	11	54–55	12.2	0.8488	1.4702	+10.5
12	11	55	12.0	0.8517	1.4701	+12.9
13	11	55–56	3.6	0.8532	1.4708	+ 4.6
14	11	56–57	1.4	0.8507	1.4725	−10.3
15	11	57–58	1.0	0.8488	1.4724	−27.7
16	11	58–59	0.5	0.8454	1.4731	−40.4
17	11	59–61	0.5	0.8435	1.4740	−48.7
18	11	61–63	0.6	0.8425	1.4753	−51.4
19	11	63–66	0.4	0.8457	1.4781	−40.7
20	11	66–68	0.8	0.8497	1.4817	−16.7
21	11	68–69	1.3	0.8530	1.4832	− 3.8
22	11	69–75	0.8	0.8508	1.4802	− 1.2
23	11	75–90	0.5	0.9176	1.4828	− 3.7
24	11	90–93	0.4	0.9292	1.4921	− 4.8
25	11	93–106	1.0	0.9389	1.4930	+ 0.2
26	11	106–112	0.6	0.9393	1.4981	+13.4
27	11	112–116	1.3	0.9358	1.4902	+24.3
28	11	116–118	2.3	0.9302	1.4973	+39.2
29	11	118–119	4.8	0.9273	1.5011	+40.0
30	11	119	2.0	0.9258	1.5020	+28.8
31	11	Above 119	3.9	0.9135	1.5042	−24.0
Pot residue and losses			2.7			

Pinus ponderosa from Beulah, Colorado (420 Gm. used)

				d_4^{24}	n_D^{25}	$[\alpha]_D^{24}$
1	10	39–40	0.6	0.8550	1.4642	+ 1.1
2	10	40–41	3.3	0.8561	1.4657	− 2.3
3	10	41–43	2.9	0.8575	1.4683	− 6.5
4	10	43–45	1.4	0.8598	1.4703	−11.6
5	10	45–46	3.5	0.8617	1.4723	−13.9
6	10	46–47	1.4	0.8620	1.4732	−14.4
7	10	47–48	4.6	0.8639	1.4738	−13.0

TABLE I.—(*Continued*)

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
8	10	48–49	3.2	0.8595	1.4734	− 8.2
9	10	49–50	2.3	0.8580	1.4734	− 4.9
10	10	50–51	2.9	0.8587	1.4730	− 0.5
11	10	51–52	8.9	0.8569	1.4726	+ 4.1
12	10	52	10.0	0.8573	1.4721	+ 8.3
13	10	52–53	9.2	0.8573	1.4718	+11.2
14	10	53	10.0	0.8584	1.4715	+14.3
15	10	53	10.0	0.8588	1.4713	+ 9.2
16	10	53–54	2.1	0.8595	1.4721	−15.1
17	10	54–56	1.5	0.8573	1.4730	−42.7
18	10	56–57	0.8	0.8451	1.4747	−63.7
19	10	57–59	1.0	0.8440	1.47742	−75.9
20	10	59–63	0.8	0.8492	1.4770	−63.7
21	10	63–65	0.5	0.8496	1.4808	−35.1
22	10	65–66	0.4	0.8647	1.4837	−17.7
23	10	66–67	1.3	0.8695	1.4855	− 4.6
24	10	67–68	0.7	0.8566	1.4833	− 1.4
25	10	68–80	1.0	0.8617	1.4881	− 6.1
26	10	80–93	0.9	0.9015	1.4801	− 8.6
27	10	93–106	0.8	0.9347	1.4863	− 0.6
28	10	106–112	0.8	0.9381	1.4875	+12.7
29	10	112–114	0.9	0.9347	1.4941	+32.0
30	10	114–115	0.6	0.9318	1.4981	+39.8
31	10	115–116	2.7	0.9322	1.5018	+46.4
32	10	116	2.3	0.9325	1.5028	+46.1
33	10	Above 116	3.1	0.9218	1.5045	+ 6.3
Pot residue and loss			2.8			

Fraction 21 had physical properties which indicated the presence of terpinolene. Two grams of the fraction were treated with bromine using the procedure of Berry (1). 2.53 Gm. of crude tetrabromide were obtained. After four recrystallizations from ethyl acetate, 0.45 Gm. of crystals, m. p. 116–117°, were received. The melting point was not depressed by admixture of known terpinolene tetrabromide. Terpinolene amounted only to 1% of the total oil. About 15% of the original charge boiled above 105° at 10 mm. and consisted mainly of sesquiterpenes. Distillation data indicated the presence of two sesquiterpenes, which occurred in highest concentration in fraction 29 and fraction 31.

Fraction 29 had the following physical properties: d_4^{25}, 0.9326; n_D^{24}, 1.5019; $[\alpha]_D^{24}$ +40.7. A 3-ml. portion of fraction 29 was used to prepare a hydrochloride. After two recrystallizations from glacial acetic acid, 1.2 Gm. of crystals which melted at 59 to 59.5° were received. An admixture of authentic hydrochloride of *d*-longifolene did not depress the melting point of the hydrochloride.

Fraction 31 had the following physical properties: d_4^{25}, 0.9134; n_D^{24}, 1.5052; $[\alpha]_D^{24}$ −40.5. A 3-ml. portion of fraction 31 was used to prepare 0.41 Gm. of hydrochloride. After two recrystallizations from glacial acetic acid, 53 mg. of hydrochloride (m. p. 119–119.5°; α_D^{25} −39, c = 1.05 in chloroform) were received. An admixture of authentic cadinene hydrochloride did not depress the melting point.

To sum up, turpentine from the Laramie Mountains, southeastern Wyoming, contained: *l*-α-pinene, 2%; *l*-β-pinene, 31%; β-myrcene, 4%; *d*-Δ^3-carene, 40%; *l,dl*-limonene, 4%; terpinolene, 1%; longifolene, 10%; cadinene, 5%.

Pinus ponderosa from Northwestern Nebraska. Oleoresin was obtained from trees growing at the easternmost locality of distribution of *P. ponderosa*, near the town of Chadron, Nebraska, at an elevation of 3,500 feet. The oleoresin yielded 18.9% of turpentine, which had the following physical characteristics: d_4^{24}, 0.8695; n_D^{24}, 1.4781; $[\alpha]_D^{24}$ −0.9°. A 3.65-Gm. batch of turpentine was distilled through the Todd column at 11 mm. pressure. A reflux ratio of 10:1 was maintained.

Fractions 1 to 13, as in the previous samples, were studied by means of the Sutherland *d/n* diagram (7).

Fractions 1 and 2 consisted of *l*-α-pinene (2% of the total oil). Fractions 3 to 9 contained mainly *l*-β-pinene (32%). Fractions 11 to 13 were those of α-Δ^3-carene (38%). β-Myrcene (3%) was identified in fraction 11 by preparing a maleic anhydride adduct, according to the method outlined by Berry (1).

The physical properties of fraction 18 indicated the probable presence of *l,dl*-limonene. Two grams of the oil were treated with bromine, as outlined previously (1). The twice recrystallized tetrabromide had a melting point of 124.5°, which was not depressed by admixture of authentic *dl*-limonene tetrabromide.

The properties of fraction 21 indicated the presence of terpinolene. The fraction was redistilled over sodium at atmospheric pressure. Two milliliters of a heart cut were treated with bromine using the procedure of Berry (1); 4.39 Gm. of crude tetrabromide were received. After four recrystallizations from ethyl acetate, 0.36 Gm. of a tetrabromide, m. p. 117.5 to 118.5°, was received. The

melting point was depressed by admixture with known terpinolene tetrabromide but the melting point was not depressed on admixture of the tetrabromide (m. p. 117.5 to 118.5°) obtained from fraction 20 of the turpentine from Beulah, Colorado (see next sample).

It should be noted that both fraction 23 of *P. ponderosa* from Beulah, Colorado, and fraction 21 of *P. ponderosa* from Chadron, Nebraska, were redistilled at atmospheric pressure over sodium. This is probably the reason for the discrepancy in results.

About 15% of the original charge boiled above 106° at 11 mm. and consisted mainly of sesquiterpenes. The distillation data indicated the presence of two sesquiterpenes, which occurred in highest concentration in fraction 29 and fraction 31.

Fraction 29 (17.6 Gm.) had the following properties: d_4^{20}, 0.9273 $n_D^{25.5}$, 1.5011; $[\alpha]_D^{25}$ +40.0. A 3-ml. portion of fraction 29 was used to prepare a hydrochloride. After two recrystallizations from glacial acetic acid, 1.3 Gm. of crystals melting at 58 to 59° were received. An admixture of authentic *d*-longifolene hydrochloride showed no melting point depression.

Fraction 31 (14.4 Gm.) had the following properties: d_4^{20}, 0.9135; $n_D^{25.5}$, 1.5042; $[\alpha]_D^{23}$ −24.0. A 3-ml. portion of fraction 31 was used to prepare 0.51 Gm. of crude hydrochloride. After two recrystallizations from glacial acetic acid, 70 mg. of hydrochloride (m. p. 119 to 120°; α^{23}, −40, c = 1.36 in chloroform) were received. When known cadinene hydrochloride of negative rotation was added, the melting point was not depressed.

To sum up, turpentine obtained from northwestern Nebraska, contained: *l,dl*-α-pinene, 2%; *l*-β-pinene, 32%; β-myrcene, 3%; *d*-Δ³-carene, 38%; *l,dl*-limonene, 7%; longifolene, 12%; cadinene, 4%.

Pinus ponderosa from Southeastern Colorado.—The oleoresin was collected near the settlement of Beulah, some 35 miles southwest of Pueblo, Colorado. Elevation of the place was 7,500 feet. Upon distillation, the oleoresin yielded 20.0% of turpentine. The turpentine possessed the following physical properties: d_4^{23}, 0.8661; n_D^{23} 1.4760; $[\alpha]_D^{23}$ +1.8°. A 420-Gm. batch of turpentine was distilled in the above described Todd column at 10 mm. pressure. A reflux ratio of 10:1 was maintained.

Physical properties of fractions 1 to 15 were studied following Sutherland's method (7). Fractions 1 to 3 consisted of slightly dextrorotatory α-pinene, (about 5%), apparently with an admixture of *l*-α-pinene. Fractions 4 to 9 were *l*-β-pinene fractions (about 17%). Fractions 12 to 15 were composed of *d*-Δ³-carene (about 40%). Fractions 10 and 11 apparently contained β-myrcene.

Berry's procedure (1) was carried out with 10 Gm. of maleic anhydride and 10 Gm. of fraction 11; 150 mg. of adduct, m. p. 33.5 to 34.5°, were obtained. The melting point was not depressed by admixture with the maleic anhydride adduct of known β-myrcene.

A sudden change in rotation in fraction 16 and a rapid increase in a levorotatory compound, with a peak of −64.0 in fraction 19, indicated the presence of *l,dl*-limonene in fractions 16 to 22. Two milliliters of the fraction were treated by the method of Berry (1). 1.14 Gm. of crude tetrabromide were received. After several recrystallizations from ethyl acetate, alternated with chloroform and addition of cold methanol, a few mg. of crystals melting at 103 to 104° were received. The melting point was not depressed by admixture of authentic *dl*-limonene tetrabromide. Limonene amounted to 15% of the total oil.

Fraction 23 had properties which indicated the presence of terpinolene. The fraction was redistilled at atmospheric pressure over sodium. Two milliliters of a heart cut were treated with bromine using the procedure of Berry (1). 3.04 Gm. of crude tetrabromide were received. After four recrystallizations from ethyl acetate, 0.47 Gm. tetrabromide, m. p. 117.5 to 118.5°, was received. The melting point was depressed by admixture with either terpinolene tetrabromide or the tetrabromide of *l*,β-phellandrene. Apparently, terpinolene was absent in this sample. About 11% of the original charge boiled above 106° at 10 mm. pressure and consisted mainly of sesquiterpenes. The distillation data indicated that the main sesquiterpene constituent was most pure in fraction 31.

Fraction 31 had the following physical properties: d_4^{24}, 0.9322; n_D^{25}, 1.5018; $[\alpha]_D^{24}$ +46.4. A 3-ml. portion of fraction 31 was used to prepare a hydrochloride. After two recrystallizations from glacial acetic acid, 1.6 Gm. of crystals which melted at 58.5 to 59° were received. When authentic longifolene was added, the melting point was not depressed.

Fraction 33 had the following physical characteristics: d_4^{24}, 0.9218; n_D^{25}, 1.5045; $[\alpha]_D^{24}$ + 6.3. The fraction was redistilled over sodium, producing a 7.0 Gm. heart cut with the properties: d_4^{23}, 0.923; n_D^{23}, 1.5039; $[\alpha]_D^{23}$ +13.7. A 3-ml. portion of the heart cut was used to prepare a hydrochloride. After two recrystallizations from glacial acetic acid, 0.21 Gm. of hydrochloride, m. p. 59 to 59.5°, was received. The melting point was not depressed by admixture of authentic hydrochloride of *d*-longifolene.

To sum up, the turpentine sample from southeastern Colorado was composed of the following ingredients: *dl*-α-pinene, 5%; *l*-β-pinene, 17%; β-myrcene, 10%; *d*-Δ³-carene, 40%; *l,dl*-limonene, 15%; longifolene, 8%.

The results of the analyses of the five samples of turpentines, together with seven samples previously reported (2–5) show that *Pinus ponderosa* turpentine, throughout the extensive range of the distribution of this species, always contained large amounts of Δ³-carene; within the species there are several chemical varieties or forms. Penfold (6) calls these physiological varieties. In some of these varieties, monocyclic terpenes were represented by limonene, in others by limonene and terpinolene.

In some varieties the sesquiterpenes were represented by longifolene and cadinene; in other varieties by cadinene alone or by longifolene alone.

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Composition of Gum Turpentine of Pines XXX*

A Report on *Pinus serotina, Pinus tenuifolia,* and *Pinus yunnanensis*

By N. T. MIROV

Pinus serotina **turpentine was found to consist of *l*-α-pinene, 5 per cent; *l*-limonene, 90 per cent; methyl chavicol, 1 per cent; and 2 or 3 per cent of unidentified sesquiterpenes. *P. tenuifolia* turpentine contained: *d*-α-pinene, 77 per cent; *l*-β-pinene, 6 to 7 per cent; Δ³-carene, 8 per cent; terpinolene, 1 to 2 per cent; methyl chavicol (possibly) less than 1 per cent; linalool, about 1 per cent; sesquiterpene fraction, 1 per cent. *P. yunnanensis* turpentine contained *l*-α-pinene, 87 per cent; possibly small amounts of *l*-β-pinene; oxygenated terpene compounds, 5 or 6 per cent. Apparently there were no sesquiterpenes.**

Pinus Serotina **Mich.**—*Pinus serotina*, or pond pine, sometimes is described as a subspecies (1) or a variety of *Pinus rigida*. Shaw (7) and Little (5) consider this pine as an independent species.

Pinus serotina grows on the coastal plains from southern New Jersey to central and northwestern Florida and Alabama. Its turpentine was analyzed in 1908 by Herty and Dixon (3). The steam distilled turpentine had these physical characteristics: density, d^{20} = 0.8478; index of refraction, n_D^{20} = 1.4734; specific rotation, $[\alpha]_D^{20} = -105°36'$. The turpentine began to boil at 172°. In the fraction boiling at 175° to 176°, Herty and Dixon identified *l*-limonene by preparation of a tetrabromide. No other terpenes were identified, although a small amount of sesquiterpenes was suspected. Herty and Dixon reported that 90 per cent of the turpentine consisted of *l*-limonene.

In 1954 we received a sample of pond pine oleoresin from the Olustee Experimental Forest, northern Florida. The turpentine was obtained by heating the oleoresin under reduced pressure; at the end of the operation, the pot temperature reached 212°, and the pressure was reduced to 2 mm. The yield of turpentine was 19 per cent of the weight of the oleoresin. The physical characteristics of the turpentine were these: density, d_4^{25} = 0.8437; index of refraction, n_D^{25} = 1.4716; specific rotation, $[\alpha]_D^{25}$ = −83.7.

A 300-Gm. batch of the turpentine was fractionated with a Vigreux-type column, 80 cm. long and 1.5 cm. inside diameter, equipped with a heated jacket. A reflux ratio 10 to 1 was maintained. The pressure was 663 mm. In fractions 1 and 2, α-pinene was identified. A copious precipitate of pinene nitrosochloride was obtained from both fractions when the oil was diluted with glacial acetic acid, chilled, mixed with amyl nitrite, and a 1:1 mixture of hydrochloric and acetic acids was added dropwise. After three recrystallizations from chloroform by addition of cold methanol, the nitrosochloride melted at 104–105°.

Attempts to identify β-pinene in fraction 6 were not successful. Physical characteristics of fractions 8 to 17 (Table I) clearly indicated the presence of large amounts of *l*-limonene. This terpene was identified in fraction 13 by preparation of limonene tetrabromide. After four recrystallizations from ethyl acetate, the tetrabromide possessed a melting point of 105–106°. Fraction 17 was extremely fragrant. It contained a great deal of limonene, to be sure, but also, apparently, an admixture of some oxygenated compounds. This fraction was redistilled to remove most of limonene. The higher boiling part of the fraction (1.3 Gm.) was oxidized to homoanisic acid. [See THIS JOURNAL **43**, 741(1954)]. Recrystallized from hot *n*-hexane, the homoanisic acid possessed a m. p. of 85–86°. The homoanisic acid was further oxidized, by the action of chromic anhydride, to anisic acid. Recrystallized from *n*-hexane, the acid had a melting point of 183.7–184.6°. There was no depression of melting point upon admixture of authentic anisic acid. Thus, presence of methyl chavicol in the turpentine of *P. serotina* was established.[1] Possibly the flask residue contained some sesquiterpenes, but these did not amount to more than 2 or 3% of the total oil.

To sum up, turpentine of *P. serotina* contained: *l*-α-pinene, 5%; *l*-limonene, 90%; methyl chavicol, 1%, and 2 or 3% of unidentified sesquiterpenes.

Pinus Tenuifolia **Benth.**—*Pinus tenuifolia* Benth. was discovered and named in 1839, but later it lost its specific rank and was described by Shaw (7) as a variety of *Pinus pseudostrobus*. Martinez (6) considers it an independent species.

Judging by the chemistry of its turpentine, which is much more complicated and quite different from that of *P. pseudostrobus*, we concur with Martinez, and consider *P. tenuifolia* a valid species.

An oleoresin sample of *P. tenuifolia* came to us through the courtesy of Mr. Louis Huguet from the vicinity of Uruapan, Michoacan, Mexico. The turpentine was expelled from the oleoresin under reduced pressure; at the end of the distillation, pot temperature was 180°, and pressure was 2 mm. The yield of turpentine was 27%. The physical characteristics of the turpentine were as follows: density, $d_4^{23.5}$ = 0.8600; index of refraction, $n_D^{23.5}$ = 1.4672; specific rotation, $[\alpha]_D$ = +25.6.

A batch of 964 Gm. of the turpentine was fractionated in the Todd apparatus described in previous articles of this series; 72% of the turpentine was

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Included in this article is a brief summary of the entire project.

[1] Thanks are due to Dr. Gene Kritchevsky for identification of methyl chavicol.

distilled under atmospheric pressure, the rest under a pressure of 9 mm. Hg. The results of the fractional distillation are shown in Table I.

The lower-boiling fractions consisted of *d*-α-pinene; this terpene was identified in fraction 2 by the usual preparation of nitrosochloride, which after several precipitations from chloroform by cold methanol, possessed a melting point of 105 to 106°. Fraction 12 was tested for β-pinene, by the usual preparation of nopinic acid, which, after two recrystallizations from benzene, had a melting point of 127°.

Fraction 16 yielded a nitrosate; after recrystallization from chloroform by addition of cold methanol,

TABLE I.—FRACTIONAL DISTILLATION OF TURPENTINE OF PINUS SEROTINA, P. YUNNANENSIS, AND P. TENUIFOLIA

Fractions	Pressure	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
(1) *Pinus serotina* (308 Gm. used)				d_4^{25}	n_D^{25}	$[\alpha]_D^{25}$
1	663	150–161	1.7	0.8445	1.4617	−42.0
2	663	161–162	4.0	0.8416	1.4681	−49.8
3	663	162–163	1.9			
4	663	163–164	9.5	0.8423	1.4689	−57.9
5	663	164–165	2.2			
6	663	165–166	14.6	0.8426	1.4693	−70.6
7	663	166–167	1.3			
8	663	167–168	2.2	0.8402	1.4702	−88.0
9	663	168–169	16.6	0.8397	1.4706	−100.0
10	663	169–170	4.7			
11	663	170–171	5.5	0.8396	1.4709	−108.4
12	663	171–172	7.8			
13	663	172–173	14.6	0.8396	1.4714	−117.3
14	663	173–174	2.3			
15	663	174–175	1.3	0.8427	1.4725	−111.7
16	663	175–178	1.7			
17	663	178–200	1.9	0.8713	1.4829	−85.0
Residue	...	...	4.9	...	...	...
Losses	...	...	1.3	...	...	...
(2) *Pinus tenuifolia* (964 Gm. used)				d_4^{25}	n_D^{25}	$[\alpha]_D^{25}$
1	760	107–155	0.4			
2	760	155–156	62.0	0.8557	1.4641	+40.4
3	760	156–157	9.7	0.8558	1.4651	+35.1
4	760	38–39	2.6	0.8569	1.4662	+29.9
5	769	39–41	1.1	0.8579	1.4673	+22.8
6	769	41–43	0.4	0.8588	1.4681	+18.9
7	769	43–44	0.8	0.8593	1.4697	+8.2
8	769	44–45	0.5	0.8595	1.4710	−0.2
9	769	45–46	0.8	0.8595	1.4721	−7.0
10	769	46–47	0.9	0.8602	1.4733	−13.7
11	769	47–48	1.0	0.8602	1.4740	−17.0
12	769	48–49	1.2	0.8602	1.4740	−17.0
13	769	49–50	1.3	0.8602	1.4740	−14.0
14	769	50–52	1.4	0.8602	1.4731	−4.6
15	769	52–53	1.2	0.8593	1.4721	+3.5
16	769	53–54	3.5	0.8584	1.4719	+13.5
17	769	54–55	2.3	0.8581	1.4712	+14.5
18	769	55–56	0.5	0.8581	1.4730	+7.4
19	769	56–62	0.7	0.8508	1.4750	−0.9
20	769	62–66	0.6	0.8548	1.4800	−2.1
21	769	66–68	0.5	0.8614	1.4843	−0.9
22	769	68–70	0.3	0.8626	1.4850	−1.2
23	769	70–80	0.4	0.8711	1.4782	−2.5
24	769	80–84	0.6	0.8711	1.4681	−12.1
25	769	84–86	0.5	0.8711	1.4685	−12.0
26	769	86–90	0.3	0.8964	1.4745	−9.4
27	769	90–95	0.8	0.9433	1.4841	−8.9
Residue and losses		...	3.7	...	...	...
(3) *Pinus yunnanensis* (29 Gm. used)				d_4^{25}	n_D^{25}	$[\alpha]_D^{25}$
1	760	153–154	3.8	0.8535	1.4630	−47.3
2	760	154–155	61.3	0.8510	1.4638	−50.5
3	760	155–157	13.1	0.8523	1.4648	−47.7
4	760	157–159	5.9	0.8556	1.4664	−42.1
5	760	159–161	2.4	0.8530	1.4683	−32.2
Residue	...	above 161	7.6	0.8743	1.4823	−6.9
Losses	...	...	5.9	...	...	...

the nitrosate melted at 146°. Thus the presence of *d*-Δ^3-carene in turpentine of *P. tenuifolia* was established.

Terpinolene was detected in fraction 21. A tetrabromide prepared from the oil of this fraction was recrystallized from chloroform by addition of cold methanol. The melting point of the tetrabromide was 109 to 110°.

Fractions 23 and 24 possessed a fragrant odor, suggesting the presence of an oxygenated terpene compound. Fraction 24 was examined by Dr. Gene Kritchevsky and was found to contain linalool. Its *p*-nitrobenzoate possessed a melting point of 69.0 to 70°. Its phenylurethane melted at 63.6 to 64.2°. The spectral data of fraction 24 suggested the presence of small amounts of methyl chavicol.

Judging from the density of fraction 27, it obviously contained some sesquiterpenes. A hydrochloride was prepared using 3 Gm. of the oil of this fraction, dissolved in 24 cc. of dry ether, and treated with dry HCl gas for thirty minutes at 0°. The ether was then evaporated. Upon standing in a refrigerator for three weeks, hydrochloride crystals were formed, but these melted as soon as the evaporating dish was removed from the refrigerator.

Similar behavior of a hydrochloride melting at low temperature has been observed in *Pinus insularis* and *P. glabra*.

To sum up, turpentine of *P. tenuifolia* contained: *d*-*α*-pinene, 77%; *l*-*β*-pinene, 6 to 7%; *d*-Δ^3-carene, 8%; terpinolene, 1 to 2%; methyl chavicol (possibly), less than 1%; linalool, about 1%; sesquiterpene fraction, 1%.

***Pinus yunnanensis* Franchet.**—*Pinus yunnanensis* was described by Franchet in 1899 as an independent species. Shaw (8) considers it as a variety and calls it *Pinus sinensis* var. *yunnanensis*. Lately, however, the name *P. sinensis* has fallen into misuse and to a certain degree has been replaced by the name *P. tabulaeformis*. *Pinus yunnanensis* has again become in the eyes of some botanists (4) a valid species.

Wu (9), working with herbarium material, arrived at the conclusion that *Pinus yunnanensis* and *Pinus insularis* are the same species. It is seen then, that opinions differ as to the botanical status of *P. yunnanensis*.

This pine grows in all of Yunnan province except the alpine zone and in the southwestern part of Kweichaw province. It also occurs in upper Burma, northern Viet Nam, and in a narrow belt of northern India.

As it was difficult to obtain oleoresin of this pine from its native habitat, the writer tapped a few planted trees growing in the Eddy Arboretum, Institute of Forest Genetics, near Placerville, California. A little more than 30 Gm. of turpentine was obtained by heating the oleoresin under reduced pressure. At the end of the distillation, temperature was raised to 180°, and the pressure was reduced to 1 mm.

The yield of turpentine was 22.6%; its characteristics were as follows: density, $d_4^{21.6} = 0.8591$; index of refraction, $n_D^{23} = 1.4663$; specific rotation, $[\alpha]_D^{23} = -44.3$.

A batch of 29 Gm. was fractionated in a Todd column fitted with a Vigreux-type tube 80 cm. long and 1.5 cm. inside diameter. The reflux ratio was 10 to 1 throughout the distillation. Results of the fractionation are presented in Table I.

Judging from the physical characteristics of the fractions, *P. yunnanensis* turpentine consisted of at least 87% *l*-*α*-pinene. A pinene nitrosochloride was prepared from fraction 2. The nitrosochloride, recrystallized from chloroform by addition of cold methanol, possessed a melting point of 106–107°. Addition of authentic pinene nitrosochloride did not lower the melting point.

A slight drop in density of fraction 5 suggested the presence of another terpene, but there was not enough material to identify it.

Characteristics of the residue (which was not "oily" and was very fragrant) do not point to any appreciable amounts of sesquiterpenes, but they suggest the presence of a dextrorotatory oxygenated compound.

To sum up, *P. yunnanensis* turpentine contains *l*-*α*-pinene, 87%; possible *l*-*β*-pinene (small amounts), and oxygenated terpene compounds 5 or 6%. Apparently there were no sesquiterpenes.

ENTIRE PROJECT SUMMARY

This paper completes the project of systematic investigation of chemical composition of gum turpentines of the species of genus *Pinus*. Turpentines of 66 pines have been investigated or, in some instances, re-examined.

Some of the results of our inquiry are given.

We have found ethyl caprylate in the turpentine of *Pinus edulis*. Previously this ester was positively identified only in fusel oil of grape brandy.

A sesquiterpene, albicaulene, a corresponding sesquiterpene alcohol, albicaulol, and a diterpene, cembrene, were all three found in *Pinus albicaulis* turpentine and named. These are apparently new substances, not reported previously in any plants. Later we found albicaulene and albicaulol in several more pines.

A bicyclic sesquiterpene which was found in *Pinus pinceana* turpentine and which was named maderene is also probably a new sesquiterpene. A diterpene occurring in *P. koraiensis* turpentine which formed maleic anhydride adduct, is probably a newly found hydrocarbon.

Longifolene was reported by us in turpentines of many American and Mexican pines, often in large quantities.

We have found methyl chavicol in gum turpentines of several pines.

Linalool was identified in turpentine of *Pinus tenuifolia*.

We have found that paraffin hydrocarbons are not so rare as ingredients of pine gum turpentines as had been previously supposed; *n*-heptane

was found in turpentines of 8 pines; *n*-undecane in 7.

Myrcene was found in turpentines of 5 pines and ocimene in 1. Terpinolene was detected in turpentines of 5 pines.

A bicyclic terpene, Δ^3-carene, proved to be a rather common terpene in American pines. Some varieties of *Pinus ponderosa* contained more than 50 per cent of this terpene.

Besides contributing to knowledge of turpentine chemistry, information reported in the publications of this series has served as a foundation for studying the distribution, variability, and inheritance of terpenes and associated nonterpene compounds of the species of the genus *Pinus*.

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Concentration of an Alfalfa Growth Factor for *Neurospora sitophila* and Its Use in the Microbiological Assay of Pyridoxine*

By ARTHUR F. NOVAK, MARY LOU JONNARD, and JOSEPH A. LIUZZO†

Dehydrated alfalfa leaf meal contains an unidentified factor(s) necessary for maximum growth of *Neurospora sitophila* in Difco Bacto Pyridoxine Assay Medium. A procedure for one-thousand-fold concentration of this factor was developed which involved extraction of dehydrated alfalfa leaf meal with dilute hydrochloric acid, precipitation of inert material with ethanol, removal of impurities soluble in diethyl ether, chloroform, and benzene, followed by chromatographic adsorption of the active substance on fuller's earth, and then elution with hydrochloric acid. Supplementation of 100 Gm. of Difco Bacto Pyridoxine Assay Medium with approximately 0.5 Gm. of concentrate will improve use of the medium for the microbiological assay of pyridoxine in vitamin products containing alfalfa concentrates.

IN A PREVIOUS PUBLICATION (1) in 1953, it was shown that the addition of alfalfa extracts caused excessive growth of *Neurospora sitophila* in a medium considered complete for the microbiological assay of pyridoxine. This method cannot be employed for accurate pyridoxine assays of vitamin products containing alfalfa concentrates because they contain another factor necessary for maximum growth of the test organism. The nature of this substance was studied.

EXPERIMENTAL

Dehydrated alfalfa leaf meal was used as the source of all fractions discussed in this investigation.

Difco Bacto Pyridoxine Assay Medium (Formula revised July 1954) (2), patterned after that described by Stokes, *et al.* (3), and modified by Barton-Wright (4), was employed because it is highly standardized and results can be duplicated reasonably. The fact that this commercial product is deficient in an unidentified factor(s) present in alfalfa, but which is required for maximum growth of the assay organism *Neurospora sitophila* 299 ATTC 9276, is the basis for this investigation. Any significant growth response by this organism above that obtained with the basal medium, and caused by supplementation with alfalfa or its fractionation products, was a verification of the presence of an essential substance in the fraction being tested.

Difco Bacto Neurospora Culture Agar (2) was used for maintaining a stock culture of the organism, and for preparing the inoculum. This culture was transferred at fifteen-day intervals and stored under refrigeration at 0°. To prepare an inoculum of *Neurospora sitophila*, a transfer was made forty-eight hours prior to the assaying period.

Pyridoxine stock solution was prepared by dissolving 20 mg. of pyridoxine in 200 ml. of distilled water. This standard solution was discarded after one month, and a new solution prepared. It was stored under refrigeration in an amber bottle to prevent decomposition or inactivation. The concentration of pyridoxine used per assay flask (volume 10 ml.) was 0.1 mg.

To rehydrate the assay medium, 5 Gm. Difco

* Received September 25, 1957, from the Department of Agricultural Chemistry and Biochemistry, Louisiana State University, Baton Rouge, La.
† Department of Animal Husbandry, Michigan State University, East Lansing, Mich.

Bacto Pyridoxine Assay Medium were dissolved in 100 ml. of distilled water and boiled for several minutes. The medium was cooled, and distributed in 50-ml. Erlenmeyer flasks. Five milliliters of the assay medium and 1 ml. of pyridoxine stock solution were added to each flask. This is referred to as the basal medium. Volume in the control flasks was adjusted to 10 ml. by the addition of 4 ml. of distilled water. Alfalfa concentrates to be tested were then added to the remaining flasks in concentration equivalent to 0.8 Gm. of dehydrated alfalfa leaf meal per flask. Preliminary experiments showed this to be the concentration necessary for maximum stimulation of the organism in the basal medium. This was ascertained from tests made by adding different levels of an aqueous extract of dehydrated alfalfa leaf meal to flasks containing the basal medium. Growth response of *Neurospora sitophila* was measured by comparison of mycelial weights. Results are given in Table I.

TABLE I.—GROWTH RESPONSE TO INCREASING CONCENTRATIONS OF ALFALFA

Concn. of Alfalfa, Gm./Flask	Dry Mycelial Wt.* mg./Flask
None	48.0
0.2	54.0
0.5	65.2
0.8	76.2
1.6	76.1

* Each figure is average of six flasks. Least significant difference at 1% level is 2.9 mg.

Fractions failing to cause increased growth of the organism above the control were retested in higher concentrations.

All test flasks were autoclaved at 121° for fifteen minutes, cooled to room temperature, and inoculated with a culture of *Neurospora sitophila*. Inocula was prepared by transferring one loop of spores from a forty-eight hour culture of the organism into 100 ml. sterile 1% saline. One drop of this suspension was added to each flask from a sterilized 1-ml. pipet, and the flasks were incubated at 30° for five days. At the termination of the incubation period, the mycelia were removed from the flasks with a stiff wire needle, and pressed dry between paper towels. Each mycelium was then dried to constant weight at 100° and weighed.

All pH adjustments were made with hydrochloric acid and sodium hydroxide.

This general procedure was employed throughout the entire experiment to measure the activity of the various alfalfa fractions.

Chemical and Physical Properties of the Unidentified Growth Factor(s).—This phase of the project was planned to obtain facts upon which a reasonable commercial procedure for concentration of the factor could be based. The following alfalfa fractions were made in order to show these properties. Every time a separation was obtained, each fraction was tested for growth promoting activity. Fractions with questionable activity were eliminated.

Dehydrated alfalfa leaf meal was extracted with distilled water in a 1-Gm. to 5-ml. proportion by autoclaving the mixture at 121° for thirty minutes. Another portion of leaf meal was extracted with 0.1 *N* hydrochloric acid by the same method. The mixtures were cooled and filtered through Whatman No. 1 paper. Both extracts were adjusted to pH 4.5 and tested for activity. Residues from these extractions were discarded.

The acid extract, which was found to be more active than the aqueous extract, was adjusted to pH 7.2 and the whole extract was precipitated by the addition of 2 volumes of 95% ethanol. The precipitate was separated by filtration through Whatman No. 1 paper and dissolved in water. Ethanol was removed from the filtrate by boiling. Both the aqueous solution of the precipitate and the filtrate fractions were tested for activity.

The fact that the factor(s) is extracted in acid solution under high temperature is verification of its stability in solution at a low pH. To determine whether or not the factor(s) is stable to heat in alkaline solution, the ethanol filtrate was adjusted to pH 12.0 and boiled for fifteen minutes.

Twenty-milliliter aliquots of the ethanol filtrate were adjusted to pH 4.5 and each extracted by three repeated extractions in a separatory funnel with 30-ml. portions of selected solvent. Solvents used for extractions were: diethyl ether, chloroform, carbon tetrachloride, and benzene. A 20-ml. aliquot of the ethanol filtrate was adjusted to pH 8.5 and extracted with diethyl ether in the same manner. The solvent from each extraction was evaporated to dryness on a steam bath, and 4 ml. of distilled water was added to each flask to dissolve the residue remaining in the container.

Twenty-milliliter portions of the ethanol filtrate were adjusted to various pH's and passed through 200 x 18-mm. chromatographic columns packed half full with the adsorbant. The adsorbants used were: activated carbon (Darco), fuller's earth (Sargent), and levitated alumina (Schaar). Each adsorbant selected was mixed half and half with Celite prior to packing in the column. Columns packed with the adsorbants were wetted with water before the active solutions were passed through. After passing the filtrate through the columns, each was eluted with 0.1 *N* hydrochloric acid.

The ethanol filtrate at pH 8.5 was precipitated by acetone and this precipitate dissolved in water. Acetone was removed from the filtrate by evaporation on a steam bath, and 4 ml. of distilled water was added to the residue.

After the solutions were adjusted to pH 4.5, the solvent extracts, aqueous preparations from the solvent extractions, fractions that passed through columns, and the heat treated ethanol filtrate at pH 12.0 were tested for activity. Results are listed in Table II.

To prepare a concentrate of the active substance, 100 Gm. of dehydrated alfalfa leaf meal were extracted with 500 ml. of 0.1 *N* hydrochloric acid by autoclaving for thirty minutes at 121°. This preparation was filtered through Whatman No. 1 paper, and the residue washed with distilled water before being discarded. The filtrate and washings were combined and concentrated to 500 ml. by direct heat. After adjusting the concentrated filtrate to pH 7.2, two volumes of 95% ethanol were added, stirred, and allowed to stand overnight. A gelatinous precipitate formed which was removed by

TABLE II.—SOME PROPERTIES OF THE UNIDENTIFIED GROWTH FACTORS

Supplements to Basal Medium, Equivalent to 0.8 Gm. Alfalfa	Dry Mycelial Wt.,[a] mg./Flask
None	47.5
Ethanol filtrate of the acid extract	74.0
Heat treated ethanol filtrate, pH 12.0	65.0
Extractions of ethanol filtrate:	
1. Diethyl ether, pH 4.5	
(*a*) Extract	No growth
(*b*) Aqueous phase	87.3
2. Diethyl ether, pH 8.5	
(*a*) Extract	48.0
(*b*) Aqueous phase	67.0
3. Chloroform, pH 4.5	
(*a*) Extract	47.1
(*b*) Aqueous phase	73.2
4. Carbon tetrachloride, pH 4.5	
(*a*) Extract	46.4
(*b*) Aqueous phase	85.1
5. Benzene, pH 4.5	
(*a*) Extract	47.9
(*b*) Aqueous phase	85.1
6. Acetone, pH 8.5	
(*a*) Precipitate	61.5
(*b*) Filtrate	No growth
Chromatographic columns of:	
1. Alumina levitated filtrates	
pH 4.7	70.3
pH 7.0	69.4
pH 9.0	48.1
2. Fuller's earth filtrates	
pH 4.5	62.1
pH 7.0	46.7
pH 8.5	47.4
3. Fuller's earth eluate, pH 8.5	65.0
4. Activated carbon, pH 6.0	61.7

[a] Each figure is average of six flasks. Least significant difference at 1% level is 2.6 mg.

filtration, washed with 95% ethanol, and discarded. Ethanol was removed from the filtrate by evaporation on a steam bath, and the volume was adjusted to 500 ml. by addition of distilled water. (One milliliter is equivalent to 0.2 Gm. of dehydrated alfalfa leaf meal.) This solution is referred to as the ethanol filtrate fraction.

Twenty milliliters of the ethanol filtrate was adjusted to pH 4.5 and extracted three times with 30-ml. portions of diethyl ether. The ether extracts were discarded, and the remaining solvent in the aqueous phase was adjusted to 20 ml. by the addition of distilled water. This aliquot was designated as Fraction *A*.

Fraction *A* was extracted three times with 30-ml. portions of chloroform at pH 4.5. An emulsion formed, but upon standing separated into two separate phases. The solvent extracts were discarded, and the remaining solvent in the aqueous phase was adjusted to 20 ml. with distilled water, and this was called Fraction *B*.

Fraction *B* was extracted three times with 30-ml. portions of benzene at pH 4.5. The solvent extracts were discarded and the remaining solvent present in the aqueous phase was removed by steam evaporation. Volume of the aqueous phase was adjusted to 20 ml. as above, and this solution was called Fraction *C*.

Fraction *C* was adjusted to pH 8.5 and passed through a chromatographic column of fuller's earth. Previously it was found that the active substance is adsorbed on the column at this pH. The column was eluted with 0.1 *N* hydrochloric acid and the acid eluate was concentrated on a steam bath. This solution was called Fraction *D* and is the final concentrate.

To evaluate each fraction in terms of increased growth of *Neurospora silophila* in Difco Bacto Pyridoxine Assay Medium, each fraction was tested by comparison with standard controls. To eliminate the possibility that an insufficient quantity of the active substance was present in the final concentrate, it was also evaluated at double concentration. Activity was measured according to the procedure previously discussed, and the results are presented in Table III. A schematic outline of the fractionation procedure is given in Fig. 1.

TABLE III.—ACTIVITY OF ALFALFA CONCENTRATES ON THE GROWTH OF *Neurospora silophila*

Additions to Basal Medium, Equivalent to 0.8 Gm. Alfalfa	Dry Mycelial Wt.,[a] mg./Flask
None (pyridoxine free)	17.5
None (containing pyridoxine)	48.0
0.1 *N* HCl Extract	88.5
Ethanol filtrate of the acid extract	76.0
Fraction *A*	87.0
Fraction *B*	85.0
Fraction *C*	71.5
Fraction *D*	71.9
Fraction *D* (double concentration)	70.1

[a] Each figure is average of six flasks. Least significant difference at 1% level is 2.4 mg.

An aqueous solution of the concentrated material remained stable upon exposure to light seventy-two hours and exhibited some fluorescence when exposed to ultraviolet light.

DISCUSSION

A substance causing increased growth of *Neurospora silophila* in Difco Bacto Pyridoxine Assay Medium has been concentrated from dehydrated alfalfa leaf meal. Various properties of the factor have been established during the concentration procedure.

It was shown experimentally that the growth factor(s) was extracted to a greater extent with 0.1 *N* hydrochloric acid than with distilled water. Since the acid extract was concentrated by direct heating and this fraction exhibited growth stimulation, it was established that the factor is stable to heating in acidic solution. The ethanol filtrate also showed growth activity when heated in alkaline solution. Therefore the factor is stable to heating in alkaline solution. Since ethanol was evaporated from the ethanol filtrate by direct heating at approximately neutral pH and this fraction proved to

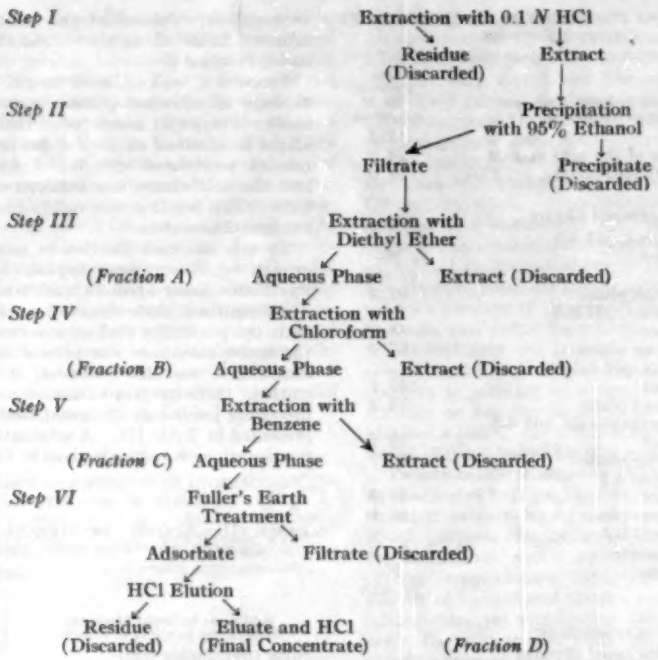

Fig. 1.—The scheme of fractionation.

be active, it was established that the substance is heat stable in neutral solutions.

The growth factor is not extracted from the aqueous phase at pH 4.5 in diethyl ether, chloroform, carbon tetrachloride, and benzene, or at pH 8.5 with diethyl ether. It is precipitated from the aqueous phase at pH 8.5 by acetone. It is adsorbed from solution at pH 9.0 by levitated alumina, and at pH 7.0 or 8.5 by fuller's earth, from which it can be eluted with 0.1 *N* hydrochloric acid. It is not adsorbed from solution at pH 4.7 or 7.0 by levitated alumina, at pH 4.5 by fuller's earth, and at pH 6.0 by activated carbon.

Growth inhibition rather than stimulation resulted when the basal medium was supplemented with either the diethyl ether (pH 4.5) extracted fraction from the ethanol filtrate, or the precipitate formed by the addition of acetone to the ethanol filtrate. This suggests the presence of an inhibitory factor for *Neurospora sitophila*. A growth depression factor was reported in the literature for chicks fed dehydrated alfalfa at high levels in a poultry ration. Lepkovsky, *et al.* (5), found that this factor could be removed from the dehydrated alfalfa by repeated water extraction which suggests that their inhibitory factor and the one described here may be the same. Evidently, a growth depression factor is either destroyed or separated from growth stimulatory factors at this point, because it was not encountered in further separations; or possibly the inhibitory factor is present in such inadequate quantities in the latter fractions that no effect is produced.

The final concentrate prepared in the laboratory contained a growth promoting factor necessary for maximum growth of *Neurospora sitophila*. This substance is either lacking in the present commercial medium or present in inadequate amounts. Since fraction *D* caused an increase in growth of 50% above the control as compared to a considerably greater stimulatory effect afforded by the alfalfa hydrolysate, it is apparent that this latter increase in growth is due to at least one additional factor, indicating a separation had been made. Verification was accomplished by failure to increase growth further with Fraction *D* by increasing the concentration used for the biological test.

The final concentrate represents less than 0.1% of the initial solids. Starting with 100 Gm. of alfalfa, the final concentrate weighed approximately 100 mg. It can be stated that 99.9% of the inactive material had been effectively removed from the dehydrated alfalfa leaf meal. Supplementation of 100 Gm. of Difco Bacto Pyridoxine Assay Medium with 0.5 Gm. of concentrate will improve its use for the microbiological assay of pyridoxine in vitamin products containing phytological concentrates.

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The Use of Active Iodine as a Water Disinfectant*

By SHIH L. CHANG

The chemistry of the formation of various species of titrable iodine in solutions of elemental iodine and iodide are described in a quantitative manner. The relative cysticidal efficiencies of various species of titrable iodine and the effect of temperature on cysticidal efficiency of I_2 are described, and equations are given for computing the cysticidal residual I_2 with a given contact time, and vice versa, at varying temperatures, as well as that for computing the cysticidal residual iodine in I_2-I_3^- systems. The relative viricidal (against Coxsackie B2 virus) efficiencies of various species of titrable iodine and the effect of temperature on viricidal efficiency of I_2 are described, and the employment of the equations used in computing the cysticidal residual iodine for I_2 and I_2-I_3^- combination, with or without modification, are given. Because of the incompleteness of the data other than those on I_2 at 25°, values obtained in various computations are presented with reservation but are considered as relatively safe for use in practice. The use of preparations of iodine for water disinfection in practice is discussed.

FROM THE AUTHENTIC information on disinfection of water by active halogens (1–7), it is apparent that of the pathogens that may normally find their way into water and water supplies, the cysts of *Endamoeba histolytica* and the enteric viruses, except the adenovirus (7), are more resistant to free chlorine and/or iodine than either the bacteria or cercariae of human schistosomes. Hence, in using active iodine as a water disinfectant, a good margin of safety is provided for the destruction of the latter organisms if the application is based on its effectiveness on the amoebic cysts and one or more of the more resistant enteric viruses.

In a quantitative sense, the destruction of microorganisms in water by active iodine is a subject of considerable complexity because of a number of reactions which elemental iodine may undergo in aqueous solutions to form various species of titrable iodine having varying germicidal efficiencies. In two previous communications the writer and his associates have reported on the usefulness of elemental iodine as a water disinfectant (3) and its use for such purpose under field conditions (8). However, in times of emergency, the iodine tablets described (8) may not be available and any of the other preparations, such as tincture of iodine, Lugol's solution, and iodophors, may have to be used, or the water to be treated may have such a high alkalinity as to break the buffering capacity of the dihydrogen pyrophosphate present in the iodine tablets, thus permitting hydrolysis of I_2, or even further changes of the hydrolysis product, hypoiodous acid. Hence, it is felt that fairly complete information on the use of active iodine as a water disinfectant should be assembled so that it can be used more efficiently under more flexible conditions.

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CHEMISTRY OF AQUEOUS SOLUTIONS OF ELEMENTAL IODINE

In various preparations of elemental iodine, iodide is used to improve its stability and solubility, except iodophors, in which the diatomic iodine is stabilized and made more water-soluble by being bound to a nonionic detergent. When a preparation of elemental iodine (excluding iodophors) is dissolved in water, the I_2 may remain in its elemental form, or it may undergo changes involving one or more reactions, depending on the initial concentration of titrable iodine and added iodide, and the pH and temperature of the solution. The formation of iodomines between ammonia or amino compounds and I_2, fortunately, does not occur under the usual conditions in water disinfection and, therefore, can be omitted from the consideration. The reactions that are of importance are as follows:

Hydrolysis of I_2—The equilibrium expression for this reaction is:

$$\frac{[HOI]\,[H^+]\,[I^-]}{[I_2]} = K_h \qquad \text{(Eq. 1)}$$

The value of the equilibrium constant, K_h, is given as 9×10^{-16} at 0° (9), and 3×10^{-13} at 25° (10–12). Since the common I_2 preparations (iodine tablets and tincture of iodine) contain enough iodide to give an I_2:I^- molar ratio of about 1:1, the per cent of titrable iodine existing as I_2 and that as HOI in aqueous solutions of elemental iodine containing 0.5 to 50 p. p. m. of total titrable iodine and enough iodide to give an I_2:I^- ratio of 1:1.2 have been computed for pH values from 5.0 to 8.0 and are presented graphically in Fig. 1. It should be noted that in the computation the formation of triiodide ion and the decomposition of HOI at pH 8.0 were ignored.

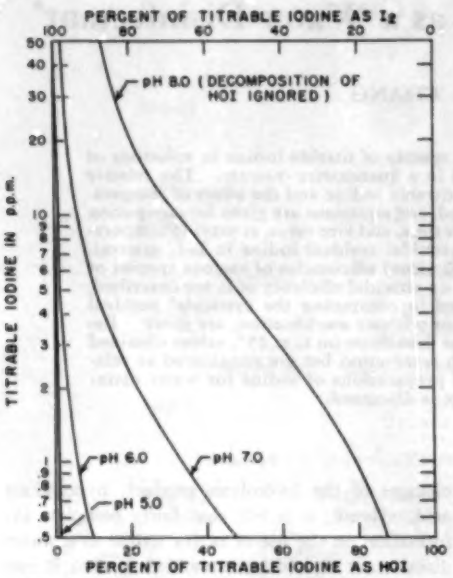
PERCENT OF TITRABLE IODINE AS I_2
100 80 60 40 20 0
50 40 30 20 10 9 8 7 6 5 4 3 2 1 .9 .8 .7 .6 .5
TITRABLE IODINE IN p.p.m.
pH 8.0 (DECOMPOSITION OF HOI IGNORED)
pH 6.0
pH 7.0
pH 5.0
0 20 40 60 80 100
PERCENT OF TITRABLE IODINE AS HOI

Fig. 1.—Per cent of titrable iodine as HOI per cent of titrable iodine as I_2 and HOI in aqueous solutions at 25°.

In Fig. 1 it is seen that in an aqueous solution of elemental iodine containing a total titrable iodine of 0.5 p. p. m. and over an enough iodide to give an I_2:I^- molar ratio of 1:1.2, an insignificant amount of I_2 is hydrolyzed until the pH rises to 6.0 or above. At a total titrable iodine concentration of 5 p. p. m. or greater, an insignificant amount of I_2 is hydrolyzed until the pH rises to 7.0 or above. Hence, for practical purposes, it may be stated that at pH 6.0 or below and total titrable iodine of 0.5 p. p. m. and above, or at pH 7.0 or below and total titrable iodine of 5 p. p. m. and above, all of the titrable iodine exists as I_2. At pH 7.0 and titrable iodine concentrations below 5.0 p. p. m., the amount of I_2 hydrolyzed ranged from about 50% at 0.5 p. p. m. to 10% at 5.0 p. p. m. At pH 8.0 the amount of I_2 hydrolyzed by computation ranges from 90% at 0.5 p. p. m. to about 20% at 20–30 p. p. m.; but the slow decomposition of HOI to form iodate upsets the computation to a greater or lesser degree, depending on the buffering system existing and the time of standing.

Dissociation of HOI.—At high pH values, 9.0 and over, the HOI formed from hydrolysis of I_2 undergoes dissociation. The equilibrium expression for this reaction is:

$$\frac{[H^+]\,[OI^-]}{[HOI]} = K_a \qquad \text{(Eq. 2)}$$

The value of K_a is given as 4.5×10^{-13} at 25° (1). From this value, it is estimated that insignificant amounts of HOI are dissociated until the pH value is raised to over 10.0. Since the HOI decomposes rapidly at pH values of 9.0 and over, it becomes apparent that the existence of OI^- ion in significant amounts is of such short duration that it has very little, if any, practical importance.

Decomposition of HOI.—At pH values over 8.0, the HOI formed from hydrolysis of I_2 is unstable and decomposes to form iodate and iodide. The reaction or reactions may be briefly expressed as follows:

$$3HOI + 2(OH^-) \rightleftharpoons HIO_3 + 2H_2O + 2I^- \qquad \text{(Eq. 3)}$$

According to the data of Wyss and Strandskov (12), the decomposition rate in a solution containing 30 p. p. m. or less titrable iodine is slow at pH 8.0 maintained by a borate or carbonate buffer, whereas in the presence of a phosphate buffer, $^2/_3$ of the HOI is gone in forty minutes. At pH 9.0 the rate of decomposition is so fast that in ten minutes about $^2/_3$, $^3/_4$, and $^5/_6$ of the HOI go into formation of iodate in borate, carbonate, and phosphate buffer, respectively. This rapid decomposition of HOI at high pH values makes it extremely difficult, if not impossible, to compute the amounts of OI^- ion formed in solutions of titrable iodine in which significant amounts of HOI are dissociated.

Formation of I_3^- Ion.—In the presence of added iodide in an aqueous solution, the I_2 goes into formation of tri-iodide ion or periodide. The formation of higher periodide (I_5^-, I_7^-, etc.) has been reported, but does not occur in dilute solutions used in water disinfection work. The equilibrium expression for the formation of tri-iodide ion is as follows:

$$\frac{[I_2]\,[I^-]}{[I_3^-]} = K_i \qquad \text{(Eq. 4)}$$

The value of K_i is given as 1.4×10^{-3} at 25° and 0.7×10^{-3} at 0° (13). Since the K_i and temperature have a semilog relationship, the values of K_i at other temperatures can be extrapolated from a curve prepared by plotting these 2 values of K_i against the reciprocal of their respective absolute temperatures on a semilog graph paper. For instance, the values of K_i at 6° and 35° have been found by this method to be 0.82×10^{-3} and 1.85×10^{-3}, respectively (14).

Equation 4 can also be written as:

$$[I_3^-] = \frac{[I_2]\,[I^-]}{K_i} \qquad \text{(Eq. 5)}$$

From Equation 5 it is seen that the amount of tri-iodide formed in acidic aqueous solutions of elemental iodine with added iodide increases with the increase in the concentration of iodide and total titrable iodine. For convenience of evaluating cysticidal and viricidal efficiencies of solutions containing both I_2 and I_3^- in the parts that follow, the percentages of these two species of titrable iodine in solutions having varying amounts of elemental iodine and iodide at I_2:I^- molar ratios ranging from 1:1.2 to 1:9.6 have been computed and are presented in Fig. 2.

Since, as stated before, the common preparations of elemental iodine, such as the iodine tablets and tinctures of iodine, contain I_2 and iodide at a molar ratio close to 1:1, it may be stated for practical purposes that in water disinfection by iodine, all of the titrable iodine exists as I_2 when its initial concentration is 20 p. p. m. or less and the pH is kept

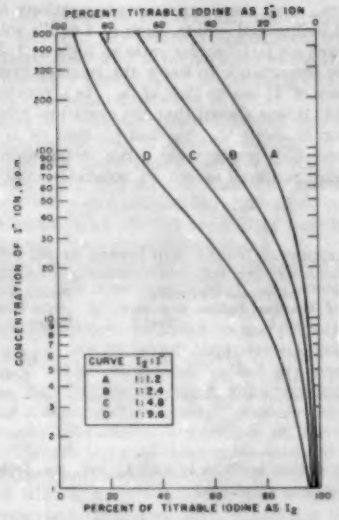

Fig. 2.—Per cent of titrable iodine as I_2 amounts of titrable iodine as I_2 and I_3^- in acidic solutions at 25° and having varying I_2:I^- molar ratios.

under 7.0. As the initial concentration of titrable iodine increases, the amount of I_3^- ion formed is correspondingly increased; but even at a concentration of 500 p. p. m. of titrable iodine, only about 50% of it goes into the formation of I_3^- ion.

CYSTICIDAL EFFICIENCIES OF VARIOUS SPECIES OF TITRABLE IODINE

Elemental Iodine.—At a given temperature and a given concentration of *E. histolytica* cysts, the minimum cysticidal residual I_2 is a function of contact time. The quantitative relationship between the cysticidal residual I_2 and contact time can be expressed by the following equation:

$$C^n t = k \qquad \text{(Eq. 6)}$$

where C is the residual titrable iodine as I_2 in p. p. m.; t is the contact time in minutes; n is the cysticidal residual I_2 concentration coefficient; and k is a constant, the value of which is dependent on the temperature and the initial cyst concentration.

The cysticidal efficiency of I_2 also increases with the rise in temperature. The quantitative relationship between k and temperature is expressed by the equation:

$$Q_{10} = \left(\frac{k_2}{k_1}\right)^{10/(T_2 - T_1)} \qquad \text{(Eq. 7)}$$

where Q_{10} is the temperature coefficient for a 10° change within the range where the temperature itself exerts no detrimental effect on cysts, and k_1 and k_2 are constants for temperatures T_1 and T_2, respectively.

With an initial concentration of 30–60 cysts per ml. of test fluid, which represents on the average a contaminated water containing 0.05–0.1 per cent fecal matter and therefore provides a wide margin of safety in disinfection work, the relationship between cysticidal I_2 concentrations and contact times has been determined at several temperatures (3, 15, 16). The results observed in these studies are summarized in Fig. 3. From this figure, the cysticidal residual I_2 required for a fixed contact time, or vice versa at any of the presented temperatures can be easily extrapolated. From the slope of, and the spaces separating these four curves, the values for n, k, and Q_{10} have been computed. The value of n was found to be 1.4 and that of Q_{10}, 1.6. The values of k at temperatures ranging from 3 to 44° are presented in Fig. 4. With these two figures and the values of n and Q_{10}, the cysticidal residual I_2 for a fixed contact time, and vice versa, at any temperature besides those shown in Fig. 3 can be conveniently computed.

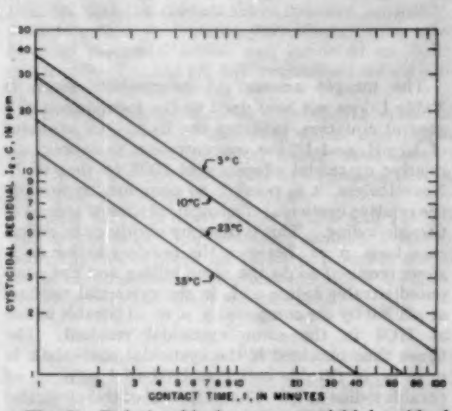

Fig. 3.—Relationship between cysticidal residual titrable iodine as I_2 and contact time at 3°, 10°, 23°, and 35° (at pH below 6.5 and I-ion concentration under 20 p. p. m.), n = slope-1.4.

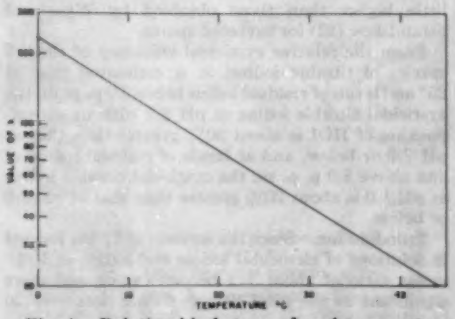

Fig. 4.—Relationship between k and temperature in destruction of amoebic cysts by elemental iodine (I_2).

Hypoiodous Acid.—The cysticidal efficiency of hypoiodous acid (HOI) has been very incompletely ascertained, mainly because of the difficulty encountered in its decomposition at pH values where significant amounts of I_2 would be hydrolyzed.

When the cysticidal efficiency of triiodide was determined (14), an experiment was carried out at pH 8.0 (with a borate buffer) with solutions of elemental iodine and iodide at an I_2:I^- molar ratio of 1:1.2. To minimize the interference caused by decomposition of HOI, a contact time of five minutes was used in the determination. The results obtained in duplicate tests made at 6 and 25° are shown in Table I.

TABLE I.—CYSTICIDAL EFFICIENCY OF SOLUTIONS OF ELEMENTAL IODINE AND IODIDE AT PH 8.0

Temp., °C	Contact Time, in Minutes	Maximum Noncysticidal Residual Iodine, p. p. m. Test 1	Test 2	Minimum Cysticidal Residual Iodine, p. p. m. Test 1	Test 2	Probable Cysticidal Residual Iodine, p. p. m.
6	5	11.9	12.5	13.7	14.5	13.1
25	5	5.8	6.3	6.9	7.2	6.6

The meager amount of information given in Table I does not lend itself to the formulation of a general equation, utilizing the hydrolysis constant of I_2, pH, and I^- ion concentration to express the relative cysticidal efficiency of HOI to that of I_2. Nevertheless, it is possible to compute empirically the relative cysticidal efficiencies of these 2 species of titrable iodine. This is done by dividing the difference in p. p. m. between the residual iodine as I_2 alone required to do the same killing and the computed titrable iodine as I_2 in the cysticidal residual at pH 8.0 by the computed p. p. m. of titrable iodine as HOI in the same cysticidal residual. The figure thus obtained is the cysticidal equivalent in p. p. m. of titrable iodine as HOI to 1 p. p. m. of titrable iodine as I_2. The reciprocal of this cysticidal equivalent of HOI to I_2 is therefore the relative cysticidal efficiency of HOI to that of I_2. The results obtained in these computations are shown in Table II. In Table II it is seen that the relative cysticidal efficiency of HOI is $^1/_3$ of that of I_2 at 6°, and is $^1/_2$ at 25°. It is of interest that these values are a little higher than those obtained by Wyss and Strandskov (12) for bacterial spores.

From the relative cysticidal efficiency of these 2 species of titrable iodine, it is estimated that at 25° and levels of residual iodine below 5.0 p. p. m. the cysticidal titrable iodine at pH 8.0 without decomposition of HOI is about 30% greater than that at pH 7.0 or below, and at levels of residual iodine of and above 5.0 p. p. m. the cysticidal titrable iodine at pH 8.0 is about 20% greater than that at pH 7.0 or below.

Triiodide Ion.—Since the amount of I_3^- ion formed in solutions of elemental iodine and iodide at I_2:I^- molar ratio of about 1:1 becomes more and more significant as the total titrable iodine rises over 20 p. p. m., and since aqueous preparations of elemental iodine and iodide, such as Lugol's solution, may have an I_2:I^- molar ratio as high as 1:3, it is of some importance to know the relative cysticidal efficiency of I_3^- ion to that of I_2. In a previous report (14) it was shown that the cysticidal efficiency of I_3^- ion is about $^1/_{11}$, $^1/_8$, and $^1/_7$ that of I_2 at 6°, 25°, and 35° respectively. For determining the cysticidal residual iodine in solutions containing titrable iodine both as I_2 and I_3^- ion, the following equation was employed:

$$R = A \frac{1 + \frac{[I^-]}{k_i}}{1 + B \frac{[I^-]}{k_i}} \qquad \text{(Eq. 8)}$$

which was used in an earlier study for determining the relative cysticidal efficiency of OCl^- ion to that of HOCl (2). R represents the total cysticidal residual iodine in p. p. m.; A is the p. p. m. of residual iodine as I_2 alone required to do the same killing; B is the ratio of the cysticidal efficiency of I_3^- ion to that of I_2; K_i is the equilibrium constant of the formation of I_3^- ion; and I^- is the molar concentration of added iodide. With this equation, the values of B at a wide range of temperature have been calculated and are presented in Table III.

TABLE III.—VALUES OF B AT TEMPERATURES FROM 0 TO 48°a

Temp., °C	Value of B	Temp., °C	Value of B
0	0.077	27	0.127
3	0.083	30	0.132
6	0.088	33	0.138
9	0.094	36	0.144
12	0.099	39	0.149
15	0.105	42	0.154
18	0.110	45	0.160
21	0.116	48	0.166
24	0.121		

a At temperatures over 48°, the cysticidal efficiency of heat itself is so high that it rapidly kills the cysts.

TABLE II.—CYSTICIDAL EFFICIENCY OF HOI RELATIVE TO THAT OF I_2

Temp., °C	Cysticidal Iodine Residual, p. p. m. at pH 8.0	Computed Titrable Iodine in p. p. m. at pH 8.0 as I_2	HOI	Computed Cysticidal Residual as I_2 Alone, p. p. m.	Cysticidal Residual Equivalent in p. p. m. for HOI:I_2	Cysticidal Efficiency of HOI Relative to That of I_2
6	13.1	10.5	2.6	11.3	3.3:1	0.3
25	6.6	3.9	2.7	5.2	2.1:1	0.5

With the values of B provided, the cysticidal residual iodine at any temperature between 0 and 48° in a given time with a preparation of elemental iodine having a known I_2:I^- molar ratio can be conveniently computed with Eq. 8.

Iodate Ion.—It has been well known that iodate is nonbactericidal. In a limited number of experiments, it was found that a solution of KIO_3 capable of liberating 10,000 p.p.m. of titrable iodine in the presence of an acid and iodide exhibited no cysticidal effect at 25° and pH 7.0 even after four hours of contact (author's unpublished data).

Iodophors.—Iodophors is a name applied to those titrable iodide compounds that are prepared by dissolving I_2 in nonionic detergents. An acid, usually phosphoric, is added to the preparation to increase its stability and improves its germicidal efficiency. The elemental iodine is supposedly loosely bound to the detergent, thus becoming more soluble in water and less volatile, corrosive, and toxic. Very little information is available on the relative germicidal efficiency of these compounds to that of I_2. From the extremely limited amount of data obtained with a preparation containing I_2 in polyethylene oxide and urea (17), it appears that the cysticidal efficiency of this compound is comparable to that of solutions of elemental iodine in which a greater portion of the I_2 is converted into I_3^-.

VIRICIDAL EFFICIENCIES OF VARIOUS SPECIES OF TITRABLE IODINE[1]

In a very recent study (18) of the viricidal efficiencies of compounds containing chlormelamine and chlormelamine-iodide combination, it was found that of the viruses in the enteric group, the Coxsackie virus (both types A and B) is definitely more resistant to these compounds than either the polio virus or Echo virus. Hence, in another study of the viricidal efficiencies of various species of titrable iodine, the Coxsackie virus (type B1) was employed. As in the preceding study (18), the production and the determination of survival of the virus were made in monkey kidney tissue cultures, and the concentration of virus was estimated by the most probable number method (19). While the current study is still underway, the data gathered thus far have been analyzed, and the results obtained are summarized here.

Elemental Iodine.—From the destruction-rate curves constructed for various concentrations of residual iodine—computed to contain 90–97% I_2 and 3–10% I_3^- at equilibrium—the contact times required for a 99.999% destruction of the virus at 25° were extrapolated and plotted against their respective I_2 residuals on a log-log scale, Fig. 5. Since there was a linear relationship between these 2 variables, Equation 6 was applied for computing the values of n and k, which came out as 0.96 and 58.5, respectively. The fact that the value of n is almost 1.0 indicates that the viricidal residual I_2 and contact time are inversely proportional to each other.

When these data are compared to those obtained for solutions of the iodine tablets used on the Lansing strain of polio virus in a previous study (3), it is noticed that the earlier data were more irregular and that there is some discrepancy in the respective viricidal efficiency findings. This, however, could be readily explained by the fact that the earlier data were obtained with crude mouse brain suspensions used in relatively large amounts in the test water.

The data on the effect of temperature on destruction of the virus by I_2 are incomplete but indicate that the value of Q_{10} may very well be greater than 2.0, which appears to be true also in destruction of the Coxsackie A2 virus by free chlorine (6).

Hypoiodous Acid.—Results on the viricidal efficiency of HOI were irregular in early experiments. As more and more data are being compiled, it appears that HOI is more viricidal than I_2, based on the fact that the contact times required for a comparable rate of destruction were shorter with solutions of elemental iodine and iodide at an I_2:I^- molar ratio of 1:2 at pH 8.5 (maintained with a borate buffer) than at pH 6.0.

Triiodide Ion.—Repeated experiments made at 25° with solutions of elemental iodine and iodide at very high molar ratios of iodide to I_2 showed that the I_3^- ion is nonviricidal at concentrations as high as 25 p. p. m. of titrable iodine.

Iodate Ion.—In tests made at 25° and pH 7.0 with solutions of KIO_3 containing as much iodate as being capable of liberating 14,500 p. p. m. of titrable iodine in the presence of an acid and iodide, no demonstrable viricidal effect was observed.

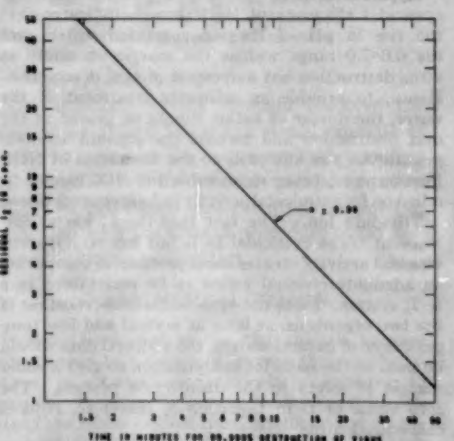

Fig. 5.—Residual I_2 concentration-time relationship in destruction of Coxsackie B1 virus in water at 25° by elemental iodine.

COMPARISON OF THE VIRICIDAL AND CYSTICIDAL EFFICIENCIES OF VARIOUS SPECIES OF TITRABLE IODINE

Elemental Iodine.—It is of importance that while the value of k for the virus destruction by I_2 at 25°

[1] The viricidal data presented in this paper were obtained in a current study carried out by Dr. Gerry Berg, virologist, at the Center.

is very close to that for the cyst destruction (58.5 *vs.* 60.0), the value of n for the former is about $^2/_3$ of that for the latter (0.96 *vs.* 1.4). Since the negative cultures in cyst survival determinations have been computed to indicate a 99.998% destruction of cysts, the cysticidal and viricidal results are comparable. These values of n and k, therefore, indicate that the increase in residual I_2 facilitates a greater cut in cysticidal than in viricidal contact time. For a given residual I_2 the viricidal contact time in the range not over forty minutes is longer than the cysticidal contact time, and also for a given contact time the viricidal residual I_2 is greater than the cysticidal residual. On the other hand, the increase in viricidal residual I_2 becomes more and more marked over the increase in cysticidal residual as the contact time is reduced toward the one-minute period. For instance, when Fig. 5 is compared with the 25° curve in Fig. 3, it is noticed that the corresponding viricidal and cysticidal I_2 residuals are 6.3 and 3.7, 13.6, and 6.0, and 34.0 and 12.5 p. p. m. for the contact times of ten, five, and two minutes, respectively.

Since the value of Q_{10} for cyst destruction by I_2 is 1.6 and that for the virus destruction, greater than 2.0, it becomes apparent that as the temperature is increased above 25°, the viricidal efficiency of I_2 increases more and more markedly than the cysticidal efficiency. Assuming that the Q_{10} value for the virus destruction is 2.5, then the viricidal residual I_2 for a ten minute contact at 35° would be 2.5 p. p. m. and that for cyst destruction, 2.2 p. p. m. The contact time for the virus destruction by 5.0 p. p. m. residual I_2 at 35° would be five minutes as against four minutes for the cyst destruction.

Hypoiodous Acid.—The higher viricidal but lower cysticidal efficiency of HOI than I_2 indicates that the rise in pH of the iodine-treated water over the 6.0–7.0 range widens the margin of safety in virus destruction but narrows it in cyst destruction. Hence, to provide an adequate treatment of the water, the dosage of iodine should be geared at the cyst destruction and include the amount to compensate for the loss of I_2 to the formation of HOI. Furthermore, being so unstable the HOI cannot be relied on for germicidal activity in long contact times.

Triiodide Ion.—The fact that the I_3^- ion at 25° is about $^1/_3$ as cysticidal as I_2 but has no apparent viricidal activity creates some problem in computing an adequate residual iodine to be maintained in a I_2–I_3^- system. Since the virus is the more resistant of the two organisms, at least at normal and low temperatures of natural waters, the viricidal data should be used as the basis for computation to give a wider margin of safety in the disinfection process. The zero value of B in Equation 8, therefore, reduces this equation to

$$R = A\left[1 + \frac{[I^-]}{K_i}\right] \qquad \text{(Eq. 9)}$$

when it is employed for computing the viricidal residual iodine in I_2–I^- systems.

Iodate Ion.—The lack of demonstrable viricidal and cysticidal effects of iodate ion at high concentrations suffices to consider it nongermicidal and indicates a lack of germicidal activity of decomposition products of HOI at high pH values.

DISCUSSION

An efficient use of halogens and active halogen compounds as water disinfectants demands a clear understanding of their chemical reactions in weak water solutions and the relative germicidal efficiency of each species of active members that may be formed in the solution. In the preceding parts the chemistry of the formation of various species of titrable iodine in water and the cysticidal and viricidal efficiencies of each species formed have been described.

Since natural fresh waters rarely contain iodide, it is felt that given the pH, temperature, and titrable iodine demand of the water, and the iodide content in the preparation of elemental iodine, it should be relatively simple with the information previously presented to compute the dosage of the iodine preparation needed to treat the water adequately for drinking purposes.

If, as appears essential, the pH of the treated water is maintained at levels not over 8.0, only the I_2, HOI, and I_3^- ion need to be considered in the computation. Since computation involving all of these three species of titrable iodine is too complicated for practical use, and since the amount of HOI formed is relatively insignificant under conditions where a significant amount of I_3^- ion is formed, and vice versa, it is calculated that an additional 10–15% allowance in titrable iodine in computing the adequate residual iodine in I_2-HOI or I_2-I_3^- systems should take care of practically all of the more complex conditions.

Since infectious hepatitis has become recently more and more significant as a water-borne disease, it appears that basing the computation of the adequate dosage of iodine preparations on the virus destruction not only provides a wider margin of safety for waters of normal temperatures but also is a necessity in areas where this disease occurs in significant numbers. However, when the water temperature is unusually high, say approaching 40°, as may be encountered in the tropics, a slightly wider margin of safety is provided when the computation is based on cyst destruction for iodine preparations containing low (not over 1:2) I_2:I^- molar ratios. When iodine preparations containing high iodide contents are used for water disinfection, the computation should be based on virus destruction at all temperatures. However, it should be noted that the palatability of the treated water must be taken into consideration in the computation. In treated waters containing more than 10 p. p. m. of residual titrable iodine, odor and taste become more of a problem. Advantage should be taken of the contact time to keep the residual titrable iodine under 10 p. p. m. At pH above the 6.0–7.0 range, the better viricidal efficiency of HOI than I_2 should be ignored.

The recommended dosage (3) of 1 iodine tablet (containing 8.5 mg. of titrable iodine) per qt. for ten minutes at water temperatures prevailing in the warm months in the temperate zone and with iodine demand not over 4.0 p. p. m., or two tablets per qt. for ten minutes at temperatures near 0–5° or at former temperatures but with iodine demand over 4.0 p. p. m., appears to be insufficient for

adequate virus destruction. On the basis of the present information on destruction of the Coxsackie virus, it seems that with either of these applications the contact time should be increased to fifteen minutes for the higher, and twenty minutes for the lower temperatures.

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Sensitivity to Vaginal Jellies*

Correlation Between Clinical Tests and Animal Tests

By JOHN H. HOLZAEPFEL, JOHN S. WARNER, JAY A. BUXTON, and JULIET A. HOWARD

A rabbit vaginal-irritation test described in this article is recommended as a reliable procedure for the evaluation of vaginal jellies and creams. The importance in a drug evaluation test of procedure, type of tissue involved, and method of interpreting the results are discussed.

IN THE DEVELOPMENT of improved contraceptive vaginal jellies, reliable tests for the irritative properties of experimental formulations are required. During the course of our development program, clearance from irritative effects was obtained for an experimental jelly (referred to here as Jelly III) by a Draize (1) rabbit-eye test and a rabbit vagina test involving gross examination of the genitalia and microscopic examination of cross-sections of the vagina. Subsequent clinical field trials revealed that the jelly produced a pronounced irritation which precluded its marketability. Since the previously applied laboratory animal tests were apparently inadequate, a program was initiated to develop a dependable animal mucosal-irritation test which could serve as a preliminary screening method to help avoid the clinical testing of any irritating vaginal preparations.

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The microscopic examinations involved in the tissue cross-section study and in the vaginal smear tests were made by M. A. Davis, Davidson Laboratories, Columbus, Ohio.

The approach to the problem involved the design of an animal test which would simulate as closely as possible the experimental procedure and conditions of the clinical vaginal-irritation test. The cytological method which we used in connection with the clinical procedure could also be included in the animal test.

EXPERIMENTAL DESIGN

Rabbits were used as the test animals because they are easy to handle and because their epithelial tissues, in general, are particularly sensitive to irritants. In order to have a fundamental knowledge of the rabbit vaginal tract, anatomic and microscopic studies were carried out. It was noted that the urinary bladder empties into the vagina via the urethral opening 2 to 3 cm. inside the introitus. The lower vagina, below the urethral opening, is somewhat wider and contains many more folds than tha upper vagina.

Histological studies made on tissue cross-sections showed that the lining of the lower vagina consists of low cuboidal basal cells and irregular squamous-type surface cells common to the urinary system. The lining of the upper vagina consists of low cuboidal basal cells and columnar surface cells common to the uterus. It was noted that the epithelial

lining is only 3 to 6 cells deep throughout the entire vaginal tract. This tissue should, therefore, be particularly sensitive; any irritant should cause an immediate reaction because of the close proximity of the irritant to the underlying capillaries, the source of inflammatory exudate.

The rabbit test was first carried out in accordance with the clinical schedule, and a special effort was made to instill the jelly high in the upper vagina above the urethral opening to prevent it from being flushed out during urination. The results of these first tests were very inconsistent and unreliable. It was found that probing the upper vagina of the rabbit triggered urination in many cases. There appeared to be enough back pressure during urination to flush out the entire upper vagina as well as the lower vagina.

Subsequent rabbit tests were carried out by applying the jelly to the lower vagina. It was found that this treatment did not trigger urination. The applications of douche and jelly, the gross observations, and the vaginal smears could be made much more easily and with less mechanical irritation. An additional advantage is the fact that the surface cells of the lower portion of the rabbit vagina are of the same type as those of the human vagina. The objections stated by Carleton and Florey (2) to the use of rabbits in a vaginal-irritation test are all avoided by treating only the lower vagina. This modified procedure gave much more reliable results. Reaction to irritating jellies often appeared within twenty-four hours. Three daily applications, with the final observations being made on the fourth day, were found to constitute a very acceptable schedule.

Vaginal smears, made before the first application and one day following the last application, were compared microscopically to determine the degree of irritation indicated by the increase in cellular material. The results were inconsistent and did not correlate well with the results from the gross observations. These shortcomings in the cytological technique can be attributed to both the difficulty in obtaining a valid quantitative smear and the inherently poor quantitative correlation between high levels or irritation and available cellular material. The vaginal smear was, therefore, eliminated as a part of the rabbit vaginal-irritation test.

METHODS

Clinical Test.—Four patients were used for each jelly tested. The patients were instructed to take a vinegar douche (1/4 cup of white vinegar in 2 quarts of water) followed by the application of the jelly with a vaginal applicator, nightly for six nights. Pelvic examinations to determine gross irritation and vaginal smears to determine cytological response were made before the first application and one or two days following the last application.

Rabbit Test.—Six rabbits, weighing 4 to 6 pounds each, were used for each jelly tested. During the few minutes required for observation and treatment, the animals were strapped to a board with their hind legs spread apart and then turned over on their backs. Gross observations were made by direct visual examination of the external genitalia and by intravaginal examination aided by an otoscope and speculum. Only rabbits that were completely free of all symptoms of vaginal irritation and sickness were used for the test. A vinegar douche (5 to 10 cc.) and then the jelly (1.0 cc.) were applied 1 to 2 cm. inside the lower vagina daily for three days. The applications were made with 3-cm. blunt-tipped soft rubber catheters which were attached to a rubber bulb for the douche and to a 10-cc. syringe for the jelly application. An effort was made to fill all of the many folds with jelly as gently as possible.

Gross examinations were made one day following each application. Irritation was classified as erythema, exudate, or edema. Each of these types of irritation was graded as a 1, 2, or 3 degree level, depending upon its severity. Erythema was considered the mildest type of irritation. Each degree of erythema (designated as E) was given an irritation count of 1; i. e., $E_1 = 1$, $E_2 = 2$, $E_3 = 3$. E_1 was characterized by a slight redness of the mucosa; E_2 was characterized by redness accompanied by occassional distinct blood vessels; and E_3 was characterized by a deep redness accompanied by numerous engorged blood vessels. The presence of an exudate, composed of pus and/or sloughed tissue, was considered more severe than erythema but less severe than edema. Each degree of exudate (designated as X) was given an irritation count of 2; i. e., $X_1 = 2$, $X_2 = 4$, $X_3 = 6$. X_1 was characterized by a few small globules of pus; X_2 was characterized by large globules of pus; and X_3 was characterized by large amounts of pus and/or sloughed tissue exuding from the vulva. Edema was considered the most severe type of irritation. Each degree of edema (designated as ED) was given an irritation count of 3; i. e., $ED_1 = 3$, $ED_2 = 6$, $ED_3 = 9$. ED_1 was characterized by a slight swelling of the vulva; ED_2 was characterized by a swelling of the vulva to nearly twice its normal size; and ED_3 was characterized by a swelling of the vulva to several times its normal size. The various irritation counts for the three days were totaled for each rabbit. (For example, a rabbit showing E_1 on the first day, E_2, X_2, and ED_1 on the second day, and E_3, X_3 and ED_3 on the third day would be given irritation counts of 1, 9, and 15 for each of the three days, respectively, making a total of 25.

The rabbits were allowed to recuperate for at least 10 days before being used for the test again.

Three experimental vaginal jellies were tested clinically and by the rabbit test. Jelly I was prepared as a blank; Jelly II contained 0.10% of an ingredient of unknown irritancy; and Jelly III contained 0.25% of that ingredient.

In the clinical test, Jelly I caused no detectable irritation, the patients reported no undue effects, pelvic examinations revealed no alteration in the vaginal mucous membrane, and the vaginal smears revealed no change in the cytological picture. Jelly II caused moderate irritation, the patients complained of some soreness of the labia, pelvic examinations revealed erythema of the vaginal mucosa, and the vaginal smears revealed an increase in purulent exudate and basal cells. Jelly III caused severe irritation, the patients complained of considerable pain after only a few applications, pelvic examinations revealed erythema, excessive vaginal discharge, and swelling of the labia, and the vaginal smears revealed an increase in purulent exudate and basal cells.

The experimental jellies were tested twice by the rabbit test. The order of irritation, as determined by gross observations (see Table I), was the same as that found by the clinical tests; i. e., I was less irritating than II, and II was less irritating than III. Also, the results from the two series of rabbit tests were found to agree with each other very satisfactorily. Five proprietary vaginal jellies and two proprietary vaginal creams were also tested by the rabbit test to determine what average irritation count would permit a product to be considered acceptable. The results (see Table I) indicate that 5 should be considered a maximum permissible average irritation count. This agrees with the results from the clinical testing of the experimental jellies in which I, having average irritation counts of 1 and 3 in the rabbit test was acceptable and II, having average irritation counts of 8 in the rabbit test, was not acceptable. It should be kept in mind, of course, that the rabbit vaginal-irritation test is intended as a preliminary screening method and not as a substitute for clinical testing.

incomplete understanding of the essentials of the test.

The selection of an appropriate test procedure depends on the use proposed for the drug being evaluated. The most significant results can be expected by the selection of a test that involves the same type of tissue as that to which the drug is to be applied in humans. Occasionally, because of convenience or because of the familiarity with certain testing facilities, a drug is evaluated by a test involving some other type of tissue. In these instances, the method of interpreting the results is particularly important. A striking example is afforded by the clearance that was obtained for the experimental Jelly III by the rabbit-eye test (this test has not been specifically recommended by Draize for evaluating vaginal preparations but has been adopted for this purpose by several testing laboratories). Although Jelly III caused moderate erythema which persisted for two days, the degree of irritation was considered insignificant because the eye returned to normal in three or four days. Subse-

TABLE I.—DATA FROM RABBIT VAGINAL-IRRITATION TEST

Vaginal Preparation	Test Series	Total Irritation Counts per Rabbit[a] — Rabbit Number 1	2	3	4	5	6	Average
Experimental Jelly I	1	0	0	0	6	0	0	1
	2	8	0	4	2	0	3	3
Experimental Jelly II	1	14	5	9	4	2	12	8
	2	3	17	3	11	9	2	8
Experimental Jelly III	1	29	20	19	11	16	12	18
	2	16	25	19	7	12	17	16
Proprietary Jelly A	1	0	0	1	0	0	0	0
	2	0	3	0	2	0	0	1
Proprietary Jelly B	1	1	2	0	0	0	0	1
	2	0	4	0	0	1	2	1
Proprietary Jelly C	1	2	1	3	3	1	0	2
	2	1	0	0	3	0	0	1
Proprietary Jelly D	1	0	9	0	0	0	5	2
Proprietary Jelly E	1	2	2	0	2	1	2	2
Proprietary Cream A	1	1	0	0	0	1	4	1
	2	0	2	2	2	0	0	1
Proprietary Cream B	1	3	5	3	0	5	13	5
	2	4	3	5	1	8	8	5
	3	13	7	4	5	0	0	5

[a] The irritation count is a measure of the various degrees of erythema, exudate, and edema observed on the days following each of the three daily applications of jelly. The counts recorded during the three daily observations were totaled for each rabbit. The highest total irritation count possible is 54.

DISCUSSION

Animal irritation tests involving the skin, eye, penis, or vagina have been used for drug evaluation studies for many years. The rabbit is commonly used as the test animal because it is easy to handle and because its epithelia and mucosa are particularly thin and susceptible to irritants.

The details of procedure in the rabbit vaginal-irritation test described above were found by trial and error to be particularly important. The application of the jelly to the upper vagina instead of to the folds of the lower vagina made the difference between an unreliable test and a reliable test. The misleading information initially obtained from a rabbit vagina test involving gross examination and tissue cross-sectioning probably resulted from an quent clinical trials showed that such an interpretation was not justified for a vaginal jelly. The usefulness or reliability of the Draize eye test is not disputed in the least. The experience described above emphasizes the importance of establishing a valid basis for interpreting test results. Such a basis in our rabbit vaginal-irritation test was established by correlating animal test results with clinical test results.

Additional tests are in progress to study the effects of composition on the irritancy of vaginal preparations.

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A Kinetic Study of Acid-Catalyzed Racemization of Epinephrine*

By LOUIS C. SCHROETER and TAKERU HIGUCHI

Racemization of epinephrine follows the first order law with respect to the substrate and appears to be catalyzed by hydrogen ion, the rate increasing very nearly directly with the free ion concentration. The heat of activation of the reaction and the specific rates at several temperatures have been determined. The results suggest that the racemization reaction may prove to be an important route for loss of biological activity of epinephrine solutions at pH values much below pH of 4.

LOSS OF BIOLOGICAL ACTIVITY of aqueous solutions of *l*-epinephrine may occur by either oxidative degradation or through changes in the steric relationship of the asymmetric carbon atom, i. e., racemization. The reactions are essentially independent of one another but occur to varying degrees depending on the solution. The purpose of this work is to present the results from the investigation of the racemization reaction from a chemical kinetic approach.

Although the racemization reaction of epinephrine has been the subject of a number of investigations (1–4), nearly all of these studies have been designed to show the stability of epinephrine under certain conditions and, for the most part, were not concerned with the kinetics of the process or predictions based upon such knowledge. Kisbye (5, 6) has investigated the rate of racemization at higher temperatures and has concluded that the racemization is a monomolecular process. This and our study at low pH values indicate that an entirely different mechanism was responsible for racemization in acid solutions.

The acid catalysis of epinephrine racemization is of such a type to suggest a Sn1 reaction in which water does not stoichiometrically enter into the reaction. This would seem to indicate that the carbonium ion never becomes completely free from the influence of the leaving water molecule. A shielding effect occurs so that the incoming water molecule enters from the opposite side to give a Walden inversion effect: (See mechanism of reaction, page 427.)

Bunton, *et al.* (7), have postulated a similar activated complex for optically active secondary butyl alcohol in perchloric acid solutions. They found the rate of racemization followed Hammett's acidity function, Ho (8), and that the rate of racemization was always twice the rate of oxygen exchange. The oxygen exchange was followed by using O^{18} enriched sec. butyl alcohol and H_2O^{18}. The logarithms of the rates of many acid catalyzed reactions have been tabulated and found to conform to $\log k = -Ho +$ constant (9). The log of the rate constant for epinephrine racemization has been found to fit this equation. The mechanism may be essentially

$$1 - E_p + H^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Activated Complex} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} H^+ + d - E_p$$

where water does not stoichiometrically enter into the equation.

Polarimetric determination of the rate constant involves the measurement of change in optical activity of the *l*-epinephrine solution with respect to time. The physical measurements show the epinephrine solution changing with time from levorotatory ultimately to zero optical activity when equilibrium is established. Considering only the physical measurements involved and the equation

$$1 - E_p \underset{k_d}{\overset{k_e}{\rightleftharpoons}} d - E_p, \text{ where } k_e = k_d$$

the following equation may be derived:

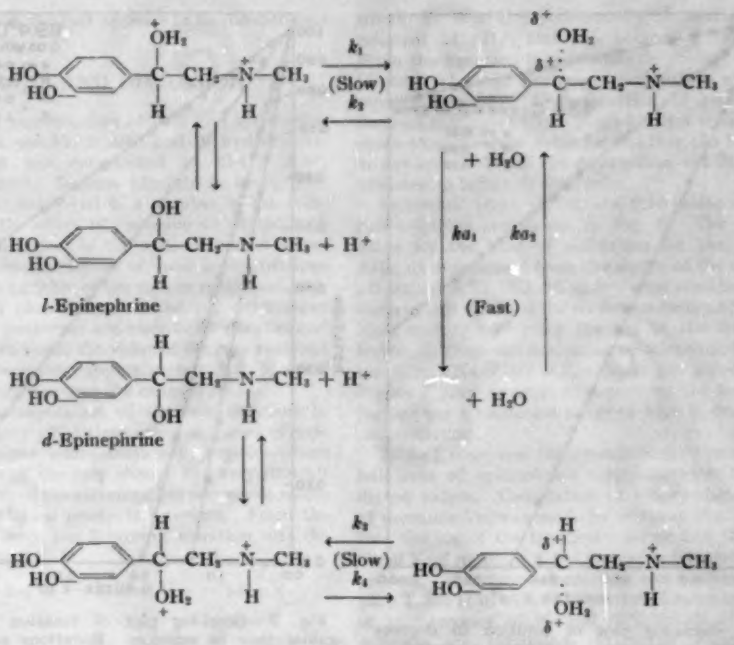
$$a = a_0 e^{-2kt};$$

where a is the rotation at any time t and a_0 is the initial rotation, the value $2k$ corresponds to the experimentally determined rate constant. This equation will describe the optical activity of a *l*-epinephrine solution at any given time under isothermal, isohydric conditions.

EXPERIMENTAL

Reagents.—Synthetic *levo*-epinephrine (Parke, Davis & Company), reagent grade perchloric acid, reagent grade hydrochloric acid, reagent grade sodium bisulfite, water redistilled from all-glass apparatus.

* Received August 1957 from School of Pharmacy, University of Wisconsin, Madison.
This study was supported in part by a contract with the Armed Service Medical Procurement Agency, Brooklyn, New York and by a grant from Parke, Davis and Co., Detroit, Mich.



Apparatus.—Zeiss Winkle Polarimeter, constant temperature bath fitted with centrifugal pump and valves, Beckman Model G pH meter with a thermostated cell.

PROCEDURE

Acid Effect and Measurement of pH.—Solutions of epinephrine were prepared by dissolving chemically assayed (10) *l*-epinephrine in standard solutions of perchloric or hydrochloric acid. Perchloric acid was employed because of its single ionization constant and nonvolatility at the experimental temperatures; it was compared with hydrochloric acid at lower temperatures to test the effect of the anion on the racemization and was found to give the same rate of racemization.

Determination of pH of epinephrine in perchloric acid solution was performed at 25° ± 1° on all solutions using the Beckman Model G pH meter calibrated against standard buffers every 0.5 pH unit over the range employed. The pH temperature coefficient of the perchloric acid solutions as determined with the glass-saturated calomel electrode system was found to introduce a small inherent error in this assumption; however, this procedure was found to give more reproducible results than that of making the determinations at the higher temperature of the experiment.

Polarimetric Determinations.—Measurements were made at 589.3 mμ (sodium D line) with the Zeiss Polarimeter fitted with filters and jacketed cells. Temperature was controlled by pumping water from an insulated thermostat with a temperature control of ±0.05° through the jacketed polarimeter cells. Flow of thermostated water through the cells was controlled by valves to limit the initial temperature time lag between cell and thermostat to two minutes and to maintain the cell at the desired temperature within ±0.05°. Three jacketed cells of various lengths and of different glass composition were employed in the study. The use of different cells served to control vessel effects. The use of the different cells in three determinations of a standard epinephrine solution gave the same rate constant.

The rate of racemization of the thermostated *l*-epinephrine solutions was determined by measuring the change in rotation with respect to time. Typical semi-log plots of optical rotation, α, against time in minutes are shown in Figs. 1, 2, 3, and 4. All of the rotations are corrected to a 2.200-dm. polarimeter tube and represent 0.05 molar epinephrine. The molar rotation, $M[\alpha]_\lambda^T$, may be calculated from any plot by using the expression:

$$M[\alpha]_\lambda^T = \alpha\ 1000/(2.200\text{ dm.} \times 0.05\text{ Molar})$$

Effect of Epinephrine Concentration and Bisulfite Concentration.—Effects of the racemization due to oxygen were minimized by using 0.1% sodium bisulfite in several solutions and determining the rate of racemization. The addition of the bisulfite did not significantly change the rate of any of the solutions under the experimental conditions of hydrogen ion concentration and temperature.

The effect of the concentration of epinephrine on the rate of racemination was tested in several determinations by employing 0.05, 0.10, 0.20, and 0.30 molar solutions of epinephrine. In this range

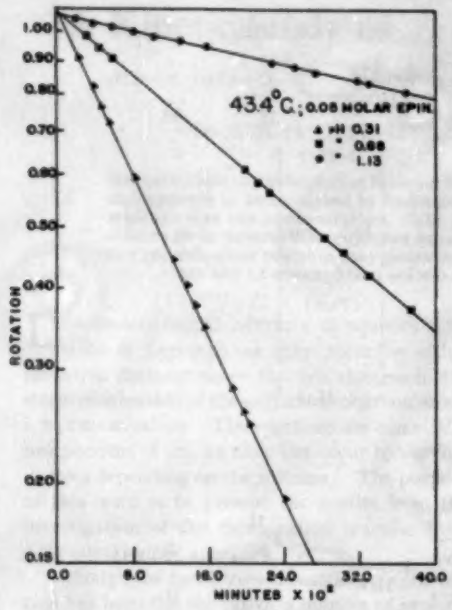


Fig. 1.—Semi-log plot of rotation in degrees against time in minutes. Rotations corrected to 2.20-dm. tube.

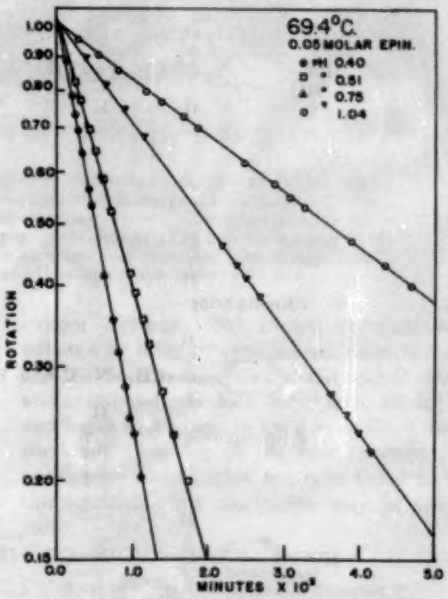


Fig. 3.—Semi-log plot of rotation in degrees against time in minutes. Rotations corrected to 2.20-dm. tube.

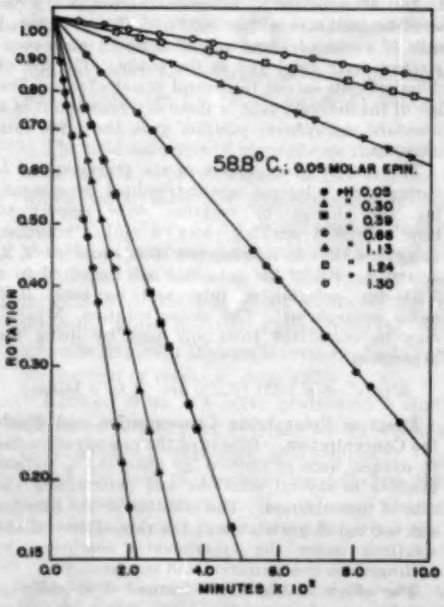


Fig. 2.—Semi-log plot of rotation in degrees against time in minutes. Rotations corrected to 2.20-dm. tube.

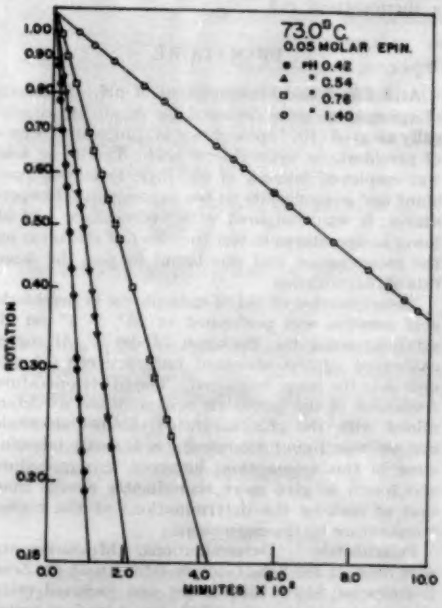


Fig. 4.—Semi-log plot of rotation in degrees against time in minutes. Rotations corrected to 2.20-dm. tube.

of concentration the rate appears to be independent of concentration.

RESULTS AND DISCUSSION

The rate of racemization of 0.05 and 0.10 molar epinephrine in perchloric acid and in hydrochloric acid solutions was determined at 43.4°, 58.8°, 69.4°, and 73.0°. Sodium bisulfite in 0.1% concentration was employed in a number of the solutions to test the effect of exclusion of oxygen and also to test the effect of the salt. Figure 5 shows the collected results of some of these determinations in which the logarithm of the rate in reciprocal minutes has been plotted against the pH determined at 25°. The isotherms are essentially parallel and have a negative slope; the value of the rate constant in minutes decreases approximately 6.2×10^{-2} per pH unit increase over the range studied.

Judicious extrapolation of the rate constant to somewhat higher pH values offers a means of prediction of isomer concentration in regions where measurements of the rate change are very difficult due to slowness of the racemization process or where colored degradation products interfere. From the experimental data the following equation was derived:

$$\log K_2 = \log K_1 - [1.21\ (pH_{(2)} - pH_{(1)})]$$

where K_1 is the experimentally determined rate constant at $pH_{(1)}$ and pH_2 is greater than pH_1. From the equation it is possible to predict the rate constant at other isothermal pH values with reasonable accuracy. Determination of several isothermal rate constants will give better results inasmuch as an average value for the rate can be determined and the log of the slope value, −1.21, can be adjusted to better fit the curve.

Arrhenius' plots of 0.05 and 0.10 molar epinephrine solutions are shown in Fig. 6. The average value for the heat of activation for the process, ΔHa, as determined from the slopes of the different pH values is 23.19 Kcal mole^{-1}; the standard deviation of this value for the six determinations is ±0.16 Kcal mole^{-1}. Plotting the log of the frequency factor, S, from the Arrhenius relationship, $\log k = \log S - \Delta Ha/2.303\ RT$, against pH gives Fig. 7. Figure 7 may be used to determine the frequency factor over a moderate range of high hydrogen ion concentration.

Table I compares the experimentally determined half-lives of epinephrine solutions with the predicted values. Calculation of the predicted rates of racemization was made by utilizing Fig. 7 to obtain the log of the frequency factor and the value 23.19 Kcal mole^{-1} for the heat of activation for the process. The same information was utilized to prepare Table II in which the predicted rates and times of maintenance of 95 and 90% levels of optical activity are tabulated. Predicted racemization rates above pH 4.0 should be regarded cautiously since this approaches the limit of reliability for the extrapolated value.

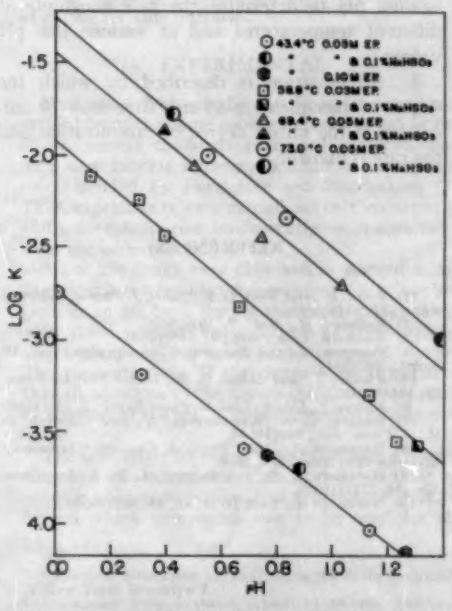


Fig. 5.—Logarithmic plots of isothermal rate constants in reciprocal minutes against pH determined at 25°. Perchloric and hydrochloric acid solutions of epinephrine employed for 43.4° isotherm; perchloric acid employed exclusively in all others. The average slope of the lines is approximately −1.208.

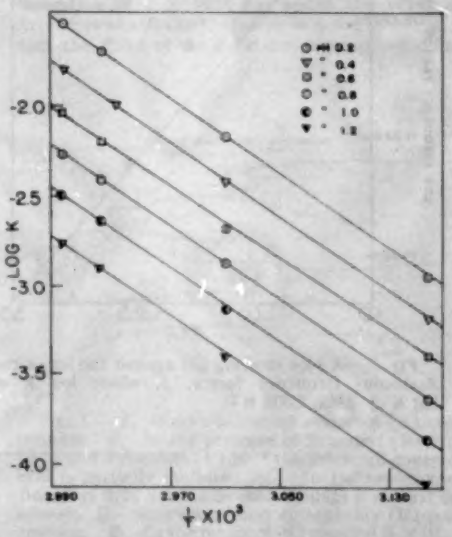


Fig. 6.—Arrhenius' plots for 0.05 and 0.10 molar epinephrine solutions. The logarithm of the rate of racemization, K, is expressed in reciprocal minutes. The average heat of activation, ΔHa, calculated from the slopes is 23.19 Kcal mole^{-1}.

TABLE I.—EXPERIMENTAL HALF-LIVES OF EPINEPHRINE AT LOW pH COMPARED WITH PREDICTED VALUES

Temperature, °C.	pH	Half-Life in Minutes By Experiment	Predicted[a]
43.4	0.77	2.75×10^3	2.75×10^3
	1.27	9.68×10^4	9.67×10^4
58.8	0.66	4.30×10^2	3.79×10^2
	1.30	2.67×10^3	2.12×10^3
69.4	0.51	7.40×10^1	8.35×10^1
	1.04	3.43×10^2	3.42×10^2
73.0	0.54	6.96×10^1	6.54×10^1
	1.40	6.90×10^2	6.32×10^2

[a] Predicted from the logarithmic form of the Arrhenius equation, $\log k = \log S - \Delta Ha/2.303\ RT$, and the half-life form for first order equations.

CONCLUSIONS

1. The racemization of epinephrine at low pH values is a first order reaction. The rate at low pH values decreases approximately 6.2×10^{-3} min.$^{-1}$ per pH unit increase. The isothermal racemization of epinephrine at different low pH values may be calculated with reasonable accuracy using a simple equation.

2. The average heat of activation for the process determined from Arrhenius' plots is 23.19 Kcal mole^{-1}. This may be used in conjunction with a plot of the Arrhenius frequency factor against pH to determine the rate constants at different temperatures and at various low pH values.

3. An equation is described by which the degree of racemization at any time may be calculated if the initial degree of racemization and the rate is known.

TABLE II.—PREDICTED RATES AND TIMES OF MAINTENANCE OF A MINIMUM 95 AND 90 PER CENT OPTICAL ACTIVITY

	Predicted Rate Constant, Min.$^{-1}$	Time of Minimum 95% Optical Activity[a]	Time of Minimum 90% Optical Activity[b]
pH 2.5, 25°	2.63×10^{-7}	1.9×10^6 min. (ca. 4½ mo.)	4.0×10^6 min. (ca. 9 mo.)
pH 2.5, 35°	9.34×10^{-7}	5.4×10^4 min. (ca. 1 mo.)	1.1×10^5 min. (ca. 2 mo.)
pH 3.0, 25°	6.92×10^{-8}	7.3×10^6 min. (ca. 17 mo.)	1.5×10^7 min. (ca. 35 mo.)
pH 3.0, 35°	2.45×10^{-7}	2.0×10^5 min. (ca. 5 mo.)	4.3×10^5 min. (ca. 10 mo.)
pH 3.5, 25°	1.91×10^{-8}	2.6×10^6 min. (ca. 60 mo.)	5.5×10^6 min. (ca. 120 mo.)
pH 3.5, 35°	6.77×10^{-8}	7.5×10^5 min. (ca. 17 mo.)	1.6×10^6 min. (ca. 36 mo.)

[a] Calculated from the following equation: $t_{95\%} = 2.303/k \log (100/95)$.
[b] Calculated from the following equation: $t_{90\%} = 2.303/k \log (100/90)$.

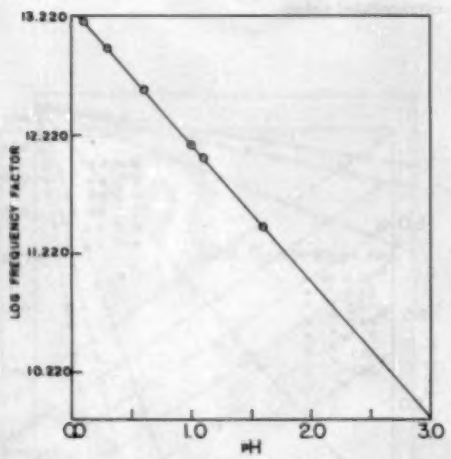


Fig. 7.—A plot showing pH against the log of the Arrhenius frequency factor, S, where $\log S = \log K + \Delta Ha/2.303\ RT$.

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A Simplified Method to Determine Adrenergic Blocking Activity of Drugs*

By EUGENE N. GREENBLATT and RICHARD K. THOMS†

A method for determining adrenergic blocking activity utilizing the responses of rabbit isolated thoracic aortic strips to L-epinephrine and L-norepinephrine has been investigated. The abilities of Priscoline, Apresoline, and Benodaine to block L-epinephrine and L-norepinephrine contractions have been determined. In general, the results obtained by this procedure agree with the conclusions reached by other investigators concerning the peripheral actions of these drugs.

FURCHGOTT AND BHADRAKOM have characterized the responses of spirally cut rabbit thoracic aortic strips to such compounds as L-epinephrine, L-norepinephrine, acetylcholine, and nitrates (1).

From their results it appeared that this method might be adapted to the determination of adrenergic blocking activity by observing the effects of drugs to be tested on contractions produced by L-epinephrine and L-norepinephrine. However, because such compounds often have diverse and sometimes antagonistic side actions it was believed necessary to test the method with known sympatholytic and adrenolytic drugs. Because of their varied potencies and side effects Apresoline[1], Priscoline,[1] and Benodaine[2] were the drugs of choice for this purpose.

EXPERIMENTAL

Methods.—Two spirally cut thoracic aortic strips excised from the same rabbit were mounted in two 30-ml. muscle chambers to record simultaneously on a slow moving kymograph under the conditions recommended by Furchgott and Bhadrakom (1). Thus, experiments were carried out on L-epinephrine and L-norepinephrine concurrently on tissues taken from the same rabbit.

All of the drugs were dissolved in normal saline. The solutions were kept frozen when not in use and kept in an ice bath during the experiment. Even with these precautions, solutions of L-epinephrine and L-norepinephrine had to be prepared weekly. The concentrations of the drugs were adjusted so that all additions to the muscle chambers were made in volumes of 0.2 ml. No temperature effects were noted.

The arterial strips were allowed to attain a state of complete relaxation and then tested for their responses to L-epinephrine and L-arterenol depending on which compound was to be used for the particular preparation. The concentration of L-epinephrine or L-norepinephrine which produced a marked contraction was the one used as the test dose for that strip.

Throughout the entire investigation these concentrations varied from 10^{-9} (Gm./ml. bath) to 10^{-8} for L-norepinephrine and from 10^{-8} to 10^{-6} for L-epinephrine. The concentrations of the drugs required to block the contractions remained constant in repeated experiments.

After relaxation from the test dose and washing, the drug to be tested for its inhibitory activity was added to the bath followed immediately by L-epinephrine or L-norepinephrine. The reversibility of the blocking actions was determined by adding the test doses of L-epinephrine and L-norepinephrine to the bath, again, after thorough washing. The only exceptions to this general procedure occurred with Priscoline.

Priscoline.—The results of a typical experiment with this drug may be seen in Fig. 1.

It was found that Priscoline itself produced contractions in the arterial strips in concentrations as low as 10^{-7}. However, when Benadryl in a concentration of 6×10^{-7} was added this effect of Priscoline was blocked. It will be noted from Fig. 1 that the shape of the Priscoline contractions differ

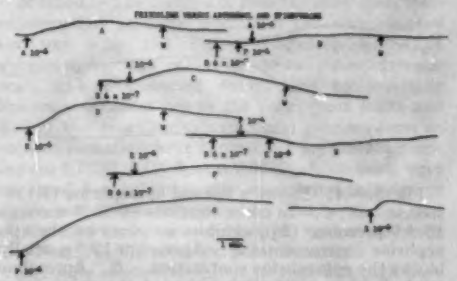

Fig. 1.—*A*. Shows a normal arterenol (A) contraction. *B*. In the presence of Benadryl (B) 6×10^{-7}, and Priscoline (P) 10^{-6} the arterenol contraction is partially inhibited. *C*. In the presence of Benadryl 6×10^{-7} arterenol exhibits a normal response. *D*. Shows a normal epinephrine (E) contraction. *E*. In the presence of Benadryl 6×10^{-7} and Priscoline 10^{-4}, epinephrine elicited a slight reversal. *F*. In the presence of Benadryl 6×10^{-7}, epinephrine exhibits a normal response. *G*. In the presence of a stimulating concentration of Priscoline 10^{-6} the epinephrine contraction was not inhibited.

* Received November 15, 1957, from the State University of New York, Brooklyn 3.

Abstracted from a thesis submitted to the Graduate School of the University of Connecticut by Eugene N. Greenblatt in partial fulfillment of the requirements for the degree of Master of Science, and presented to A.Ph.A. Convention, August 1954.

† Present address: Associate Professor of Pharmacology, School of Pharmacy, University of Connecticut, Storrs.

[1] Generously donated by Ciba Pharmaceutical Products, Inc.

[2] Generously donated by Merck and Co., Inc.

from those of L-epinephrine and L-norepinephrine being slower and more sustained.

In the presence of Benadryl 6×10^{-7} the test doses of L-epinephrine and L-norepinephrine produced normal responses, but when Priscoline in concentrations as low as 10^{-7} was also added no L-epinephrine or L-norepinephrine contractions could be elicited.

Responses to L-epinephrine and L-norepinephrine were again obtained after washing out the Priscoline and Benadryl.

These results appear to agree with the conclusions of other investigators which state, that in the rabbit, Priscoline is both histaminic and sympatholytic (2–6).

Apresoline.—The results of a typical experiment with this drug may be seen in Figs. 2 and 3.

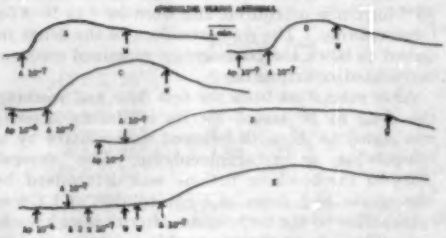

Fig. 2.—*A*. Shows a normal arterenol (A) contraction. *B*., *C*. In concentrations of 10^{-9} through 10^{-8} Apresoline (Ap) exhibits no effect on the arterenol contraction. *D*. Apresoline 10^{-6} partially blocks the arterenol contraction. *E*. Apresoline 10^{-5} blocks the arterenol contraction, and after washing, the arterenol elicits a normal response.

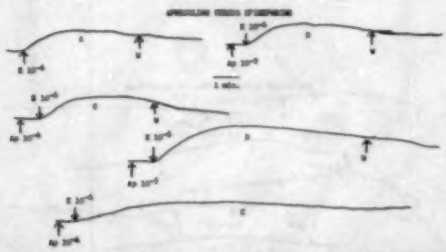

Fig. 3.—*A*. Shows a normal epinephrine (E) response. *B*., *C*. In concentrations of 10^{-9} through 10^{-6} Apresoline (Ap) exhibits no effect on the epinephrine contraction. *D*. Apresoline 10^{-6} partially blocks the epinephrine contraction. *E*. Apresoline 10^{-4} elicits a greater inhibition of the epinephrine contraction.

The contractions produced by test doses of L-epinephrine and L-norepinephrine were inhibited by a concentration of 10^{-6} of Apresoline and were completely blocked by a concentration of 10^{-4}. Responses to L-epinephrine and L-norepinephrine were again obtained after washing out the Apresoline.

The fact that these concentrations appear high when compared to those of the other drugs tested appears to agree with the results of other investigators which indicate that Apresoline has a rather low potency with regard to its sympatholytic activity (7, 8).

Benodaine.—The results of a typical experiment with this drug may be seen in Figs. 3, 4, and 5.

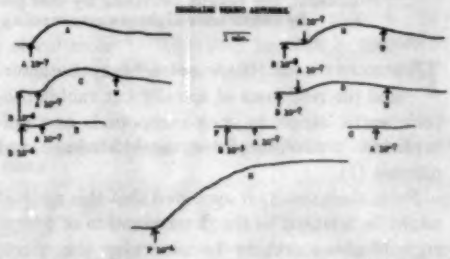

Fig. 4.—*A*. Shows a normal arterenol (A) contraction. *B*., *C*., and *D*. Benodaine (B) in concentrations of 10^{-9} through 10^{-7} had no effect on arterenol contractions. *E*. Benodaine 10^{-6} partially blocks the arterenol contraction. *F*. Benodaine 10^{-5} elicits total block. *G*. After washing, no further response could be elicited with arterenol. *H*. Priscoline (P) 10^{-4} elicits a direct muscle stimulation, after apparent irreversible block of the sympathetic receptor sites with Benodaine.

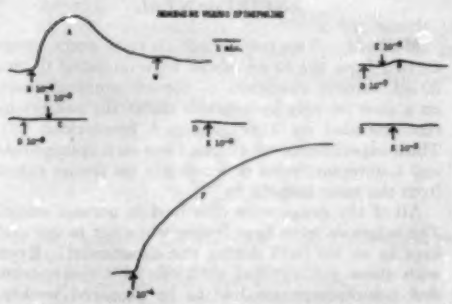

Fig. 5.—*A*. Shows a normal epinephrine (E) contraction. *B*. Benodaine (B) 10^{-9} partially blocks the epinephrine contraction. *C*. Benodaine 10^{-8} totally blocks the epinephrine contraction. *D*., *E*. Subsequent addition of epinephrine after washing elicited no response. *F*. Priscoline (P) 10^{-4} elicits a direct muscle stimulation after apparent irreversible block of the sympathetic receptor sites with Benodaine.

Concentrations of 10^{-9} markedly depressed the contractions produced by epinephrine and concentrations of 10^{-8} blocked them.

Responses to norepinephrine were inhibited by a concentration of 10^{-8} Benodaine and blocked by a concentration of 10^{-6}.

In both cases the blockades were irreversible, in that, after repeated washings the muscle would not respond again to the adrenergic stimulants. However, the muscle did exhibit responses to the histaminic actions of Priscoline.

The fact that a much higher concentration of Benodaine is required to influence the response to norepinephrine from that to epinephrine appears to agree with the literature in that Benodaine will inhibit circulating epinephrine, but will have little effect on sympathetic stimulation.

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The Polarographic Behavior of Certain Antioxidants at the Wax-Impregnated Graphite Electrode*

By ROBERT A. NASH,† DONALD M. SKAUEN, and WILLIAM C. PURDY

The anodic polarography of thirty-six phenolic compounds and nine miscellaneous antioxidants at the wax-impregnated graphite electrode is reported. Certain correlations have been made between ring substitution and half-wave potential.

THE ASSESSMENT of antioxidant properties is a matter of considerable importance to the pharmacist. Since polarography is rapid and can be conducted at low concentrations in a wide variety of media, it occurred to us that a polarographic study of a range of compounds having known antioxidant properties might prove extremely useful.

Anodic polarography has been studied far less than the cathodic variety. Although useful in some cases, the dropping mercury electrode itself readily undergoes oxidative attack, so that the stationary or rotating platinum microelectrode is often more useful (1). Gaylor and her coworkers (2–4) have recently introduced the technique of anodic polarography at the wax-impregnated graphite electrode. Lord and Rogers (5) found that the graphite electrode could be used for the anodic polarography of organic compounds. There is, however, limited data comparing the behavior of the graphite electrode with that of any other well established electrodes.

The purpose of this paper is to present polarographic data for a large number of antioxidants and related compounds. These data will be obtained on the wax-impregnated graphite electrode.

EXPERIMENTAL

Method.—The method of polarography with wax-impregnated graphite electrodes (3) was adapted for use with the Fisher "Electropode." The galvanometer was calibrated in microamperes per scale unit at selected sensitivities by inserting known resistances across the instrument leads and noting the resulting galvanometer displacement as various potentials were applied. For example, readings of 0.0257, 0.0235, and 0.0219 μA/division were obtained for galvanometer sensitivity settings of 1, 10, and 100X respectively. The potential dial of the instrument was calibrated against a Leeds and Northrup K2 precision potentiometer.

Graphite electrodes[1] fitted to cork stoppers were impregnated with Castorwax[2] at 100° for a period of two hours. The electrodes were cooled to room temperature and their exposed surfaces were coated with Seal-All.[3] A length of copper wire, serving as

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Taken in part from the Ph.D. dissertation of Robert A. Nash, The University of Connecticut, Storrs, Conn.
† Present address: Merck, Sharp and Dohme, West Point, Pa.

[1] Spectroscopic grade Graphite Electrodes of U-1 porosity and 0.25-inch diameter were obtained from the Union Carbon Products Co., Inc., Bay City, Mich.
[2] Castorwax is a product of the Baker Castor Oil Co., New York, N. Y.
[3] Seal-All is manufactured by the Allen Products Corporation, Detroit, Mich.

a lead, was fitted to the top of each electrode with a short piece of Tygon tubing and was sealed in place with a small amount of Wood's metal. This eliminated the need for a mercury contact. Fresh surfaces of the electrode were exposed for each run by cutting off lengths of between 0.12 and 0.25 inch from the bottom and abrading the exposed surfaces lightly with fine emery cloth.

Procedure.—Samples of freshly prepared test solution were taken for each run. In the case of water-soluble compounds, 10-millmolar solutions were prepared and diluted 100 times with standard Sörenson pH 7.0 phosphate buffer to obtain working concentrations of 0.1 millimolar. Where water solubility was a problem, the stock solutions were made up in alcohol and diluted with an aqueous alcohol (1:1) buffer to yield apparent pH values of approximately 7.1.

Twenty-milliliter portions of the test solution were placed in a Lingane H-cell fitted with a potassium chloride-agar plug and a saturated calomel electrode (see Fig. 1). The cell was placed in a

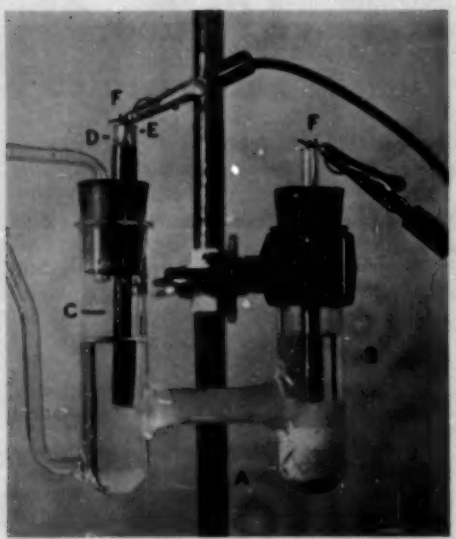

Fig. 1.—The Lingane H-Cell with a Wax-Impregnated Graphite Electrode. A.—The Lingane H-Cell. B.—The Saturated Calomel Electrode. C.—A Castorwax-Impregnated Graphite Electrode. D.—Tygon tubing. E.—Wood's metal seal. F.—Copper wire lead.

water bath thermostated at 25 ± 0.5°. Nitrogen gas was bubbled through the test solutions for fifteen minutes before each run and a nitrogen atmosphere was maintained above the solution during the course of the run. Commercial tank nitrogen was purified by passing the gas through two solutions of hypovanadate and one of test buffer before entering the H-cell. The polarograms were plotted manually.

DISCUSSION OF RESULTS

The polarographic behavior of thirty-six substituted phenols, which have been used as antioxidants, is collected in Table I. These compounds are listed in order of decreasing half-wave potential. Included, also, are the limiting currents (6) for the compounds, measured in a solution at pH 7.0 and containing a 0.1 millimolar concentration of the electroactive material. The α-values (7) for these compounds are also given.

In the case of catechol (8) and gallic acid (9), the results obtained in this study compare favorably with those obtained at a platinum electrode. Although previous studies at the graphite electrode were conducted at pH values other than 7.0, the results obtained here are in line.

The largest single class of antioxidants are the butylated derivatives of phenol. Their insolubility in water and relative lack of toxicity have made them ideally suited for the protection of fats and oils. The half-wave potentials of seven of these butylated phenols, (i.e., "Tetrabutyl Bisphenol A," 2,4-dimethyl-6-*tert*-butylphenol, 2,4,6-tri-*tert*-butylphenol, "Tenox-BHA," 2,6-di-*tert*-butylphenol, "Lederle-2246," and "Tenox-BHT"), have been presented in Table I along with the data on phenol. The half-wave potentials of *m-tert*-butylphenol, *o-tert*-butylphenol, and *p-tert*-butylphenol have been calculated to be 0.520, 0.442, and 0.468 volt *vs.* SCE at 7.0 (1, 2).

An inspection of these data indicates that butylation in the *ortho*- and *para*- positions has a profound lowering effect on the half-wave potential. *Ortho*-butylation has a greater potential lowering effect than *para*-substitution. Bis structures such as "Tetrabutyl Bisphenol A" and "Lederle 2246" offer no visible advantage in potential lowering to their monophenyl counterparts.

In Table II are found the polarographic data on nine miscellaneous antioxidants and related compounds. Again these data are in line with the work previously reported at the graphite electrode.

In a subsequent paper, correlations will be drawn between the half-wave potential and the antioxygenic efficiency.

SUMMARY

1. Polarography with wax-impregnated graphite electrodes has been adapted to study a wide variety of antioxidants and related compounds. Half-wave potentials, limiting currents, and alpha values are reported for a total of forty-five compounds.

2. Certain correlations between ring substitution and half-wave potential for phenols have been drawn.

3. The method is rapid, can be used at low concentrations in a wide variety of solvents, and should prove extremely useful for the examination of antioxidants.

TABLE I.—THE POLAROGRAPHIC BEHAVIOR OF CERTAIN SUBSTITUTED PHENOLS

Compound	$E_{1/2}$ in mv. vs. SCE	i in μA.	α	Solvent System at pH 7.0
Methylparaben[a]	759	1.28	1.46	Aqueous-alcohol
p-Hydroxpropiophenone	745	2.10	0.92	Aqueous-alcohol
p-Nitrophenol	691	3.32	1.21	Aqueous-alcohol
Phenol	537	3.22	0.81	Aqueous
Potassium guaiacol sulfonate	514	1.90	1.10	Aqueous
Vanillin	508	2.64	1.03	Aqueous
Resorcinol	490	3.32	1.05	Aqueous
p-Hydroxyphenylalanine	465	1.15	0.88	Aqueous
Hydroquinone monomethylether	352	2.20	1.25	Aqueous-alcohol
Phloroglucinol	344	2.26	0.87	Aqueous
"Tetrabutyl Bisphenol A"[b]	343	2.06	1.26	Aqueous-alcohol
N-acetyl-*p*-aminophenol	333	1.71	1.38	Aqueous-alcohol
N-*n*-butyryl-*p*-aminophenol	322	1.73	1.49	Aqueous-alcohol
β-Naphthol	310	3.14	1.26	Aqueous-alcohol
2,4,6-Tritertbutylphenol	300	0.86	1.16	Aqueous-alcohol
"Tenox-BHA"[c]	275	1.34	1.34	Aqueous-alcohol
N-*n*-pelargonyl-*p*-aminophenol	267	1.81	1.40	Aqueous-alcohol
N-*n*-stearoyl-*p*-aminophenol	267	1.93	1.44	Aqueous-alcohol
N-*n*-lauroyl-*p*-aminophenol	263	1.40	1.56	Aqueous-alcohol
2,6-Di-tert-butylphenol	208	1.09	1.09	Aqueous-alcohol
"Tenox-BHT"[d]	195	1.02	1.14	Aqueous-alcohol
"Lederle-2246"[e]	168	2.33	1.28	Aqueous-alcohol
α-Naphthol	166	3.39	1.53	Aqueous-alcohol
α-Tocopherol	140	3.50	2.12	Aqueous-alcohol
Catechol	139	1.27	1.77	Aqueous
Ethyl Hydrocaffeate	134	1.39	1.81	Aqueous-alcohol
3,4-Dihydroxyphenylalanine	119	2.45	2.03	Aqueous
Ethyl gallate	102	2.13	2.18	Aqueous-alcohol
Propyl gallate	99	2.38	2.12	Aqueous-alcohol
Nordihydroguaiaretic acid	94	1.76	2.39	Aqueous-alcohol
p-Aminophenol hydrochloride	60	1.92	1.15	Aqueous
Gallic acid	59	3.73	1.75	Aqueous
Pyrogallol	33	5.15	1.19	Aqueous
Quercetin	24	6.19	0.69	Aqueous-alcohol
Hydroquinone	18	2.24	1.31	Aqueous
Trihydroxybutyrophenone	−10	2.95	1.10	Aqueous-alcohol

[a] Methyl *p*-hydroxybenzoate, C.P., obtained from Heyden Chemical Corporation, New York, N. Y.
[b] Obtained from B. F. Goodrich Chemical Co., C.P.
[c] Butylated hydroxyanisole, food grade, obtained from Eastman Chemical Products Inc., Kingsport, Tennessee.
[d] Butylated hydroxytoluene, food grade, obtained from Eastman Chemical Products, Inc.
[e] 2,2'-methylenebis (4-methyl-6-tertbutylphenol), C. P., obtained from American Cyanamid Co., New York, N. Y.

TABLE II.—THE POLAROGRAPHIC BEHAVIOR OF SOME MISCELLANEOUS ANTIOXIDANTS AND RELATED COMPOUNDS

Compounds	$E_{1/2}$ in mv. vs. SCE	i in μA.	α	Solvent system at pH 7.0
Aniline	682	1.46	0.59	Aqueous
2,3-Diphenylindole	532	1.76	1.46	Aqueous-alcohol
Bithionol[a]	457	1.56	0.98	Aqueous-alcohol
"Dichlorophene"[b]	452	2.40	0.97	Aqueous-alcohol
Hexachlorophene	406	2.45	1.19	Aqueous-alcohol
Phenothiazine	239	3.13	0.95	Aqueous-alcohol
"Santoquin"[c]	201	1.56	2.67	Aqueous-alcohol
Rutin	199	1.43	3.81	Aqueous-alcohol
N,N'-Diphenyl-*p*-phenylene diamine	−5	3.10	1.32	Aqueous-alcohol

[a] "Actamer," U. S. P. obtained from Monsanto Chemical Co., St. Louis, Mo.
[b] Obtained from Sindar Corporation, New York, N. Y. C. P.
[c] 6-Ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline, technical, obtained from Monsanto Chemical Co.

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The Correlation of Polarographic Half-Wave Potentials of Antioxidants with Their Antioxygenic Efficiency*

By ROBERT A. NASH,† DONALD M. SKAUEN, and WILLIAM C. PURDY

The anodic polarography of forty-five antioxidants at the wax-impregnated graphite electrode has been presented in a previous paper. In this present communication, certain correlations between polarographic potentials and older potentiometric conventions for the measurement of the potential of an irreversible organic oxidation have been drawn. Furthermore, a correlation between half-wave potential and antioxidant activity is presented. Finally, recommendations are made for the use of ten antioxidants in pharmaceuticals and for eight antioxidants in commercial nonedible preparations.

THERE ARE A NUMBER of ways of quantitatively expressing the potential for an irreversible organic redox reaction. Conant and Pratt (1) introduced the A.O.P. (apparent oxidation potential) in their study of the irreversible oxidation of phenolic compounds. The A.O.P. is defined as the potential developed in thirty minutes by a solution containing the reduced form of the phenol and equimolar amounts of ferrocyanide and ferricyanide. Several years later, Fieser defined the normal oxidation potential (E_0) as the potential difference between a dilute solution of equimolar quantities of the oxidant and the reductant, when neither is ionized, and a hydrogen electrode in the same solvent (2). Subsequently, Fieser (3) defined the critical oxidation potential (E_c) as the potential obtained by an extrapolation of a potential-percentage composition plot for the reaction between the phenol and a suitable reference redox couple.

The polarographic half-wave potential ($E_{1/2}$) for a reversible redox system is equal to the standard oxidation potential minus a constant. This relationship has been shown to apply to the reversible quinone systems (4). For irreversible systems, however, the half-wave potential and the apparent oxidation potential, normal oxidation potential, and critical oxidation potential are only empirical. Since the half-wave potential is much more readily obtainable than the latter three potentials, a study into the relationship between polarographic and potentiometric potentials for systems potentially valuable in pharmaceutical preparations was undertaken.

DISCUSSION

Sixteen compounds were selected to cover a potential range of 1200 millivolts. Half-wave potentials and decomposition potentials (5) were obtained for each compound at the wax-impregnated graphite electrode by the method described in a previous paper (6). These polarographic values were converted to potentials vs. N.H.E. by means of the following formulae:

$$E_p = E_{1/2} + 0.059\ \text{pH} - (-0.246) \text{ at } 25°$$

$$E_D = E_d + 0.059\ \text{pH} - (-0.246) \text{ at } 25°$$

In Table I are collected the E_p and E_D potentials together with the corresponding literature values of E_c and E_0 for these sixteen compounds. The E_c and E_0 values reported are for solutions at pH = 7, made up in the same solvents as used in this study. Missing values indicate the lack of literature data for the particular compounds.

An examination of Table I indicates a rather close correlation between E_p and E_c and between E_D and E_0. The correlation between E_c and E_0 is not nearly so good. The relationship between E_p and E_c is accepted by all investigators in the field of polarography. The differences between the experimentally determined E_p values and the reported E_c values can be attributed to one or more of several factors, such as differences in electrode material, ionic strength of solutions, etc. The correlation

TABLE I.—THE OXIDATION POTENTIALS OF SIXTEEN SELECTED COMPOUNDS

Compounds	Potentials in mv. vs. N.H.E. E_c	E_p	E_0	E_D
p-Nitrophenol	..	1350	1433*	1276
Aniline	..	1342	1135	1173
Phenol	1225	1197	1089	1106
Resorcinol	1205	1150	1043	1051
β-Naphthol	..	970	1017	909
Phloroglucinol	..	1004	799	808
α-Naphthol	..	826	797	752
Gallic acid	799	719	..	622
Catechol	793	799	742	749
p-Aminophenol	733	720	673	619
Hydroquinone	710	678	631	634
Pyrogallol	713	683	609	624
α-Tocopherol	770	806	..	761
1,4-Naphthoquinone	484	483	..	549
l-Ascorbic acid	390	300	..	423
9,10-Anthraquinone	154	161	..	223

* Value calculated by Egloff, *et al.* (7).

* Received October 26, 1957, from the University of Connecticut, Storrs.
Taken in part from the Ph.D. dissertation of Robert A. Nash, the University of Connecticut, Storrs.
† Present address: Merck, Sharp and Dohme, West Point, Pa.

between E_D and E_e could be due to the fact that both these potentials behave like limiting potentials. E_e represents the potential at which a compound first begins its irreversible oxidation of a reversible reference redox system. E_D, on the other hand, is the potential at which a compound first undergoes reduction at an electrode surface.

Although these correlations are not absolute and are subject to such factors as choice of electrode, solvent system, pH, and ionic strength, the results of this study indicate that the half-wave potential, determined at a wax-impregnated graphite electrode, may replace the normal oxidation potential for such compounds as antioxidants. Furthermore, the critical oxidation potential may be replaced by the decomposition potential.

A comparison of the half-wave potential and the antioxygenic efficiency index for twenty-four compounds is presented in Table II. The compounds are arranged in descending half-wave potential order. The antioxygenic efficiency index for each compound was taken from data published by Bickoff (8). The index for each compound was obtained on carotene substrates in mineral oil at 75°.

Table II indicates that the most effective antioxidants are found in the potential region of 30 to 330 millivolts. Based on the results of this table, the following antioxidants are recommended for use: 1. The "Tenoxs" (butylated hydroxyanisole and butylated hydroxytoluene) for fat soluble pharmaceuticals; 2. Rutin ($E_{1/2}$ = 199 mv.), ethyl gallate, propyl gallate ($E_{1/2}$ = 99 mv.), ethyl hydrocaffeate, and nordihydroguaiaretic acid for fat soluble pharmaceuticals in which the absence of color is not important; 3. "Santoquin," "Lederle-2246"[2] ($E_{1/2}$ = 168 mv.), phenothiazine ($E_{1/2}$ = 239 mv.), 2,4,6-tritert-butylphenol ($E_{1/2}$ = 300 mv.), the N-*n*-pelargonyl ($E_{1/2}$ = 267 mv.), N-*n*-lauroyl ($E_{1/2}$ = 263 mv.), and N-*n*-stearoyl ($E_{1/2}$ = 267 mv.) amides of *p*-aminophenol, and 2,6-ditert-butylphenol ($E_{1/2}$ = 208 mv.) for commercial, nonedible, fat soluble products; 4. 3,4-Dihydroxyphenylalanine ($E_{1/2}$ = 119 mv.), N-acetyl ($E_{1/2}$ = 333 mv.), and N-*n*-butyryl *p*-aminophenols in aqueous systems on the basis of the correlation established in fat systems.

The present study suggests that anodic polarography should be a simple and rapid general technique for the preliminary assessment of the efficiency index of new potential antioxidants.

Preliminary investigations on certain other antioxidants, chiefly sulfur-containing compounds, were also begun along the lines indicated above. Although qualitative agreement was obtained, the results of these investigations were, however, more difficult to correlate both with the older potentiometric data and with antioxidant efficiency.

TABLE II.—THE CORRELATION BETWEEN ANTIOXYGENIC ACTIVITY AND THE HALF-WAVE POTENTIAL

Compound	$E_{1/2}$ in mv. vs. S.C.E.	Index for Carotene in Mineral Oil at 75°
p-Nitrophenol	691	1
Phenol	537	1
Potassium guaiacol sulfonate	514	6
Vanillin	508	3
p-*tert*-Butylphenol	468	3
Hydroquinone monomethylether	352	46
N-*n*-butyryl-*p*-aminophenol	322	190
β-Naphthol	310	18
2,4-Dimethyl-6-*tert*-butylphenol	300	188
"Tenox-BHA"[a]	275	130
"Santoquin"[b]	201	112
"Tenox-BHT"[c]	195	142
α-Naphthol	166	154
α-Tocopherol	140	160
Catechol	139	84
Ethyl hydrocaffeate	134	174
Ethyl gallate	102	165
Nordihydroguaiaretic acid	94	208
p-Aminophenol	60	92
Gallic acid	59	7
Pyrogallol	33	155
Hydroquinone	18	8
2,5-Ditert-butylhydroquinone	−558	1
2,5-Diamylhydroquinone	−579	1

[a] Butylated hydroxyanisole, food grade, obtained from Eastman Chemical Products, Inc., Kingsport, Tenn.
[b] 6-Ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline, technical, obtained from Monsanto Chemical Co., St. Louis, Mo.
[c] Butylated hydroxytoluene, food grade, obtained from Eastman Chemical Products, Inc.

SUMMARY

1. Sixteen compounds were employed to establish two relationships between four different oxidation potentials. A correlation between the half-wave potential and the normal oxidation potential and another between the decomposition potential and the critical oxidation potential was demonstrated.

2. A correlation was established between the half-wave potential and the antioxygenic efficiency index for twenty-four of the more important antioxidants. The greatest antioxygenic index for activity is found in the half wave potential region between 30 and 330 millivolts.

3. Recommendations are made for the use of ten antioxidants in pharmaceuticals and for eight antioxidants in commercial nonedible preparations. These compounds are proposed on the basis of the potential-efficiency correlation previously established.

4. The ease with which half-wave potentials can be obtained suggests their use for the prediction of the efficiency index of a newly-synthesized antioxidant.

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The Neuromuscular Activity of a Series of Bis(3-hydroxydimethylpiperidinium Bromide) Ethers*

By A. L. FISHER† and HUGH H. KEASLING

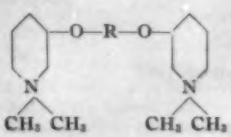
The neuromuscular blocking activity of the bis(3-hydroxydimethylpiperidinium bromide) ethers with 5–8, 10, 12, and 14 methylene groups has been evaluated in the mouse, rat, and hen. The 10, 12, and 14 methylene group derivatives were further tested in dog, rabbit, and frog in an attempt to specify their mode of blockade. Neuromuscular blocking potency increased with increase in number of methylene groups to a maximum at 12. A qualitative change in mode of action occurred with the 12 and 14 methylene compounds. It was concluded that the compounds of this series represent a mixed type of action and that the biological tests utilized do not define the activity as either competitive or depolarizing in character.

THE OBJECT of this study was an evaluation of the neuromuscular activity, mode of action, and structural relationships to activity of a series of bis(3-hydroxydimethylpiperidinium bromide) ethers. Following quantitative determinations of the neuromuscular potency of the compounds in the mouse, rat, and hen, the qualitative change in mode of action of certain of the compounds was studied further. This report details the results of this investigation.

EXPERIMENTAL

The compounds investigated were the penta-, hexa-, hepta-, octa-, deca-, dodeca-, and tetradecamethylene bis(3 hydroxydimethylpiperidinium bromide) ethers and the bis(3 hydroxypiperidinium bromide) ester of sebacic acid. The structure of these materials is detailed in Fig. 1. All compounds were dissolved in 0.8% saline, and administered as described in each experimental procedure.

Mice.—The technique of Thesleff and Unna (1) was utilized. All compounds were administered by rapid intravenous injection into the tail vein of albino mice. Paralysis was indicated by the inability of the mouse to cling to a screen inclined at 60° from the horizontal. The median paralyzing dose was calculated by the method of probits, using 13 mice at each dose level.



R = $-(CH_2)_n-$

n	*Name*	*Code No.*
5	Pentamethylene	JB 361
6	Hexamethylene	JB 367
7	Heptamethylene	JB 407
8	Octamethylene	JB 409
10	Decamethylene	JB 373
12	Dodecamethylene	JB 410
14	Tetradecamethylene	JB 418
R =	$-C-(CH_2)_8-C-$	JB 392

Fig. 1.—Chemical structure of the bis (3 hydroxydimethyl piperidinium bromide) ethers.

Rats.—The rat sciatic nerve-gastrocnemius muscle preparation as described by Van Maanen (2) was used with minor modifications. Only male hooded rats weighing from 250 to 400 Gm. were utilized. Each rat received only a single injection of drug.

Chickens.—The sciatic nerve-gastrocnemius muscle of White Leghorn hens weighing between 1.5 and 4.0 Kg. was prepared under pentobarbital

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The JB compounds utilized in this study were supplied through the courtesy of H. L. Daiell and H. Friedman. This work was supported in part by a grant from Lakeside Laboratories, Inc., Milwaukee, Wis.
† Smith, Kline & French Fellow in Pharmacology. The material contained in this report is abstracted from a dissertation submitted to the Graduate College of the State University of Iowa in partial fulfillment of the requirements for the degree of Ph.D.

anesthesia (25 mg./Kg.) as described by Pelikan, *et al.* (3). The tension on the muscle was usually 200 Gm. and held constant in each experiment. Muscle contraction was induced as described by Thesleff and Unna (1), using supramaximal voltages supplied by a Grass stimulator. The contractions were recorded by a counterbalanced lever arm on a smoked drum.

The drug effects were estimated as described by Thesleff and Unna (1). The antagonistic effects of edrophonium on the paralysis was investigated for certain of the compounds of the series by rapid intravenous injection at a dosage of 200 mcg./Kg.

The deca-, dodeca-, and tetradeca-cogeners were investigated further by the following procedures.

Dogs.—Dogs were anesthetized with 30 mg./Kg. of pentobarbital sodium. One carotid artery was cannulated and recordings made on a smoked drum via a mercury manometer. The sciatic nerve was isolated and stimulated by supramaximal shocks of 0.2 millisec. duration every ten seconds and the contraction of the anterior tibialis muscle was recorded on the smoked drum. The effects of acetylcholine, histamine, epinephrine, and bilateral carotid occlusion were determined before and after each injection of compound.

Rabbits.—Paralysis after intravenous drug administration was measured by loss of the righting response (4). The intravenous LD_{50} dosage was determined by the method of probits in groups of 13 animals and the therapeutic index was calculated for the deca-, dodeca-, and tetradeca-methylene ethers.

Cholinesterase Studies.—Cholinesterase inhibition was measured by standard manometric techniques using acetyl-β-methyl choline (0.02 M) as substrate and Winthrop-Stearns bovine erythrocyte cholinesterase (20 units/cc.). The 50% inhibitory level of the compounds tested was calculated by the method of probits.

Results.—The relative potencies of the compounds in the mouse, rat, and hen are shown in Table I.

TABLE I.—PARALYZING ACTIVITY OF BIS(3-HYDROXYPIPERIDINIUM BROMIDES)

JB No.	n	ED_{50} μmoles/Kg. Mouse	Rat	Hen
361	5	2.91	22.50	0.98
367	6	3.34	10.10	1.56
407	7	2.56	7.68	0.43
409	8	1.78	7.28	0.19
373	10	0.26	0.73	0.14
410	12	0.16	0.24	0.10
418	14	0.31	0.38	0.38
392	Sebacic acid ester	159.2	39.80	0.38
d-Tubocurarine		0.08	0.07	0.69
Decamethonium		2.1	7.4	0.015

It will be noted that the dodecamethylene compound (JB 410) is the most potent in all three species and approaches *d*-tubocurarine in potency in the curare susceptible species (rat and mouse) and is more potent than *d*-tubocurarine in the decamethonium sensitive preparation (hen). The duration of action of these materials was somewhat shorter than that of tubocurarine. The compounds having 5 through 10 methylene groups produced a decamethonium-like contracture in the hen while the dodeca (JB 410) and tetradeca (JB 418) compounds produced a *d*-tubocurarine-like paralysis.

Rabbits.—The action of the deca-, dodeca-, and tetradeca compounds on the loss of righting reflex in the rabbit was examined. As in the other species, the dodecamethylene was the most potent derivative. The intravenous LD_{50}'s for these three compounds were determined and the therapeutic index calculated. These data and a comparison of the therapeutic indices reported for *d*-tubocurarine and Laudolissin under similar experimental conditions are listed in Table II.

TABLE II.—RIGHTING RESPONSE AND TOXICITY IN THE RABBIT

Compound No.	$(CH_2)_n$	ED_{50} mg./Kg.	LD_{50}	Therapeutic Index
JB 373	10	0.160	0.360	2.2
JB 410	12	0.065	0.135	2.1
JB 418	14	0.125	0.240	1.9
d-Tubocurarine	..	...	...	1.8[a]
Laudolissin[b]	..	...	...	1.7[a]

[a] See reference (5).

[b] Decamethylenebis(1,2,3,4-tetrahydro-6,7-dimethoxyl-1-(3',4'-dimethoxy benzyl)-2 methyl isoquinolinium salts.

Antagonism.—The antagonistic effect of neostigmine or edrophonium was tested against the deca-, dodeca-, and tetradecamethylene compounds in the hen and dog. In the hen, the decamethylene compound was potentiated, the dodeca not affected, and the tetradeca was weakly antagonized by edrophonium (see Fig. 2). All three compounds were potentiated in the dog by neostigmine.

In mice, the effect of neostigmine was examined against the deca-, dodeca-, and tetradeca-methylene compounds. All were potentiated by neostigmine (see Table III).

TABLE III.—JOINT ACTION OF COMPOUNDS WITH NEOSTIGMINE IN THE MOUSE

JB No.	Dosage, mg./Kg.	Neostigmine, mg./Kg.	No. of Mice	Paralyzed, %	Dead, %
373	0.164	...	26	85	8
373	0.164	0.050	13	100	77
410	0.110	...	26	80	0
410	0.110	0.025	13	85	23
410	0.110	0.050	13	85	38
410	0.110	0.100	13	100	70
418	0.200	...	13	54	8
418	0.200	0.100	13	100	77

However, when *d*-tubocurarine chloride and the dodeca-methylene compound were tested together in mice, they had an additive effect as will be noted in Table IV.

TABLE IV.—JOINT ACTION OF *d*-TUBOCURARINE AND JB 410 IN THE MOUSE

Dosage, JB 410	*d*-Tubocurarine, mg./Kg.	No. of Mice	Paralyzed, %	Dead, %
0.100	...	13	15	0
...	0.050	13	46	0
0.100	0.050	13	100	100

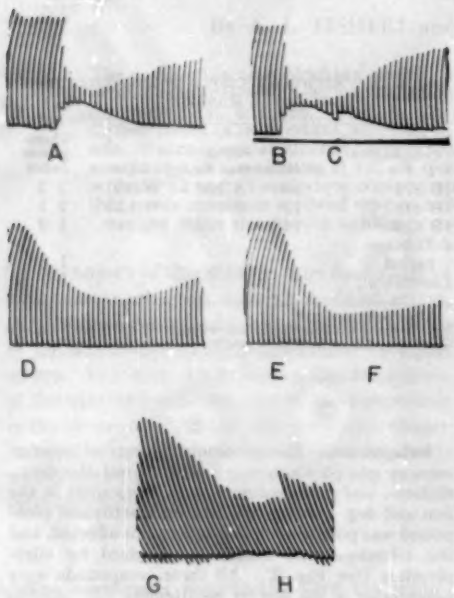

Fig. 2.—Effects of the deca-, dodeca-, and tetradeca-methylene ethers and the effect of edrophonium on the neuromuscular blocking activity of these compounds in the hen sciatic nerve-gastrocnemius muscle preparation. Injections at: A JB 373 100 μg./Kg.—B JB 373 100 μg./Kg.—C edrophonium 200 μg./Kg.—D JB 410 60 μg./Kg.—E JB 410 60 μg./Kg.—F edrophonium 200 μg./Kg.—G JB 418 200 μg./Kg.—H edrophonium 200 μg./Kg.

Dog Blood Pressure.—The deca- and dodecamethylene compounds had no effect upon dog blood pressure up to and including paralyzing doses. Both increased the depressor activity of acetylcholine fourfold which indicated the possibility of cholinesterase inhibition. There was no effect upon the pressor response of epinephrine, the depressor response of histamine, nor upon the pressor response of carotid occlusion.

Frogs.—The action of the deca- and dodecamethylene compounds was found to be at the neuromuscular junction by use of the Claude Bernard experiment. After injection of these compounds into the ventral lymph sac of frogs, the nonligated leg muscle was unresponsive to electrical stimulation of the sciatic nerve but did respond to direct electrical stimulation. The ligated leg muscle was responsive throughout the experiment to electrical stimulation of the sciatic nerve.

Cholinesterase Inhibition. Manometric studies of the inhibition of true cholinesterases by the deca-, dodeca-, and tetradeca- compounds revealed about $^1/_{100}$ the cholinesterase inhibiting activity of neostigmine. The respective molar concentrations for 50% inhibition were decamethylene (JB 373) 4×10^{-6}, dodecamethylene (JB 410) 7.8×10^{-6}, tetradecamethylene (JB 418) 5×10^{-6} and neostigmine 7.9×10^{-8}.

DISCUSSION

Numerous investigations have emphasized the importance of chain length upon neuromuscular potency. While peak neuromuscular blocking activity occurs in the decamethylene member frequently, for example, the methonium series of Barlow and Ing (6) and the isoquinolinium series of Collier (7), Smith, *et al.* (8), found in their series of bis-isoquinoliniums that the hepta- through deca- methylene derivatives were most potent in mice, rabbits, and cats but found the dodecamethylene compound most potent in the frog rectus preparation. Barlow and Ing (6) have shown that the tridecamethylene bistriethylammonium bromide is the most potent of its series. In the present series the dodecamethylene compound (JB 410) was the most potent in all species tested (see Fig. 3).

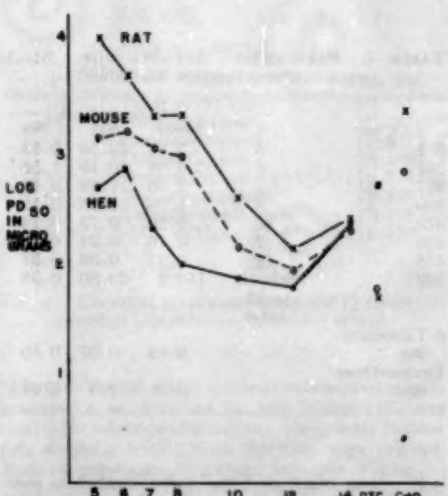

Fig. 3.—Correlation of neuromuscular paralyzing potency with length of the methylene chain.

The change from an ether linkage to an ester linkage markedly reduced the neuromuscular potency. The sebacic acid bis ester of 3 hydroxydimethylpiperidinium bromide (JB 392) was about $^1/_{800}$ as active in the mouse and rat as its corresponding ether (JB 373) but was about $^1/_2$ as active in the hen. However, it provoked contracture in the hen as did JB 373.

Of special interest was the evident change in the mode of action which occurred as the methylene chain was lengthened from the deca- to the dodecamethylene compound. The deca- and shorter chain analogs gave a definite contracture of avian muscle while the dodeca- and tetradeca- did not exhibit this effect. Further, no fasciculations were noted in the rat preparation with the dodeca or tetradeca compounds while the shorter chain members of the series all produced initial fasciculations prior to neuromuscular block. This indication of a change from decamethonium-like to a *d*-tubocurarine-like action was further confirmed by examination of the relative potencies of the deca- and tetradeca-compounds in the hen and rat. The tetradeca- was only $^1/_2$ as potent as the decamethylene in the hen which is a decamethonium sensitive species, but twice as potent in the rat which is a *d*-tubocurarine sensitive species.

Thesleff and Unna (1) reported no contracture resulted from the penta and hexamethylene members of the methonium series on the hen sciatic nerve-gastrocnemius muscle preparation whereas the higher and lower analogs did produce contracture. However, in this report the lack of contracture producing action was accompanied by a marked decrease in neuromuscular blocking activity. This was evidenced by the fact that the ED_{50} for these noncontracture producing agents was 30-fold that of the next higher analog, the tetramethonium. In our series, such large variations in potency did not occur; in fact the dodeca-, a noncontracture producing compound, was more potent than the most potent contracture provoking drug.

The results of Thesleff and Unna (1) indicated a good correlation between production of contracture in the hen and enhancement of paralysis by neostigmine in the mouse. In this series, this was confirmed by the combination of neostigmine and the decamethylene compound but not with the dodecamethylene or tetradecamethylene compounds; they stimulated no contracture yet were synergized by neostigmine in the mouse.

Zaimis (9) has reported an interesting phenomenon regarding the activity of decamethonium which she regards as evidence for a dual mode of action. She found a decreasing sensitivity of the tibialis muscle of dogs to repeated doses of decamethonium. Further, in association with this tachyphylaxis, the paralyzing effects of decamethonium were antagonized by neostigmine. We were able to confirm her results in this regard with decamethonium. However, the decamethylene compound (JB 373), the most potent drug in this series which appeared to be of the depolarizing type, was tested with the dog tibialis preparation and there was no evidence of tachyphylaxis or antagonism by neostigmine. Further, neither of these effects were noted with the dodecamethylene compound (JB 410), with the tetradecamethylene (JB 418), or with 2,5-bis(*d*-diethylaminopropylamino)-benzoquinone-bis(benzyl chloride).

At present, mode of neuromuscular blockade is generally divided into two presumptive classes, competitive or tubocurarine-like, and persistent depolarization or decamethonium-like. There is no method which can prove conclusively the exact mechanism of action. Comparison of the end plate potential of the muscle after drug administration is probably the most accurate estimation at the present time for the differentiation of mechanism of action (10). Other techniques have been used, however, which do not require such an elaborate method. Those which indicate a tubocurarine-like activity include the use of tubocurarine sensitive animals such as the rat, antagonism, and reversal of neuromuscular block by the anticholinesterases and; of course, negative responses to the indications of decamethonium-like activity which include potentiation of effect by the anticholinesterases, production of contracture in the hen, and initial fasciculation of muscle prior to the neuromuscular block.

In this series, the penta- through deca-methylene compounds were decamethonium-like in that they were more active in the hen than the rat or mouse, produced initial fasciculations in rat muscle prior to block; and all gave a contracture in the hen. In addition, the decamethylene compound, which was taken as a prototype for the shorter chain members, was potentiated by the anticholinesterases. The dodeca compound was active in both the rat and hen, was decamethonium-like in that it was potentiated by the anticholinesterases and tubocurarine-like in that it produced no initial fasciculations in rat muscle and produced no contracture in the hen. The tetradecamethylene compound appeared tubocurarine-like with respect to the increased sensitivity of rats compared to hens, the lack of contracture provoking properties in the hen while the neuromuscular block was antagonized somewhat by the anticholinesterases. In addition, it produced no fasciculations in rat muscle. However, it was decamethonium-like in regard to its potentiation by the anticholinesterases in mice.

The possibility that the action was other than at the neuromuscular end plate was eliminated for the deca- and dodeca- methylene compounds by the classic Claude Bernard frog experiment. The conclusion must be drawn then, that the compounds of this series represent a mixed type of action and that no single biological test is sufficient in itself to determine the mode of action. An examination of the effects of these mixed type agents on the end plate potentials would be of interest.

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A Report on the Oxidative Degradation of Neutralized Carbopol*

By T. W. SCHWARZ and GERHARD LEVY†

Neutralized Carbopol 934 gels undergo oxidative degradation when exposed to daylight. This reaction is probably catalyzed by trace metals. EDTA, various nonelectrolyte antioxidants and poly-hydroxy compounds may be used to retard this degradation.

AN IMPORTANT REQUIREMENT for pharmaceutical thickening agents is viscosity retention on aging. Carbopol 934,[1] a hydrophilic colloid of high thickening capacity, has been described as an agent of outstanding viscosity stability (1–4). In the course of evaluating Carbopol 934 gels in our laboratory, we have found that this polymer is subject to oxidative degradation. Carbopol 934 gels undergo a marked viscosity decrease at room temperature within two to three weeks if they are exposed to daylight.

In this paper, the stability characteristics of gels made from various salts of Carbopol 934 are reported. Some of these gels were exposed to daylight while others were not. A number of antioxidants, polyhydroxy compounds and a chelating agent were screened for their inhibitory effect on the degradation of the polymer. Viscosity measurements were used for the evaluation of the added chemicals, since the oxidative changes cause a decrease in the viscosity of Carbopol 934 gels.

EXPERIMENTAL

A 1.4% aqueous dispersion of the acid form of Carbopol 934 was prepared in a Waring Blendor. Fifty-milliliter portions of the dispersion were placed in 8-ounce glass jars. The inhibitory agent, dissolved or dispersed in 25 ml. of distilled water, was added. A sufficient amount of base in aqueous solution was then added to obtain a pH of 7.0 ± 0.1. The total solution was brought up to 100 Gm. with distilled water.

The jars were stored at room temperature (22 to 25°) in daylight, but protected from direct sunlight. Some of the jars were wrapped in aluminum foil to prevent exposure to light.

The viscosity was measured at 25° with a Brookfield Synchro-Lectric Viscometer, Model LVF, spindle No. 4 at 6 r. p. m.

RESULTS AND DISCUSSION

Table I shows the viscosity change of gels made from various salts of Carbopol 934 after 17 days of storage. While the gels which had been protected from light maintained their viscosity, the gels that were exposed to light—regardless of the neutralizing agent used—decreased in viscosity from 33% to 51%.

Table II represents the effect of light on the viscosity of Sodium Carbopol 934 gels in the presence of several antioxidants, poly-hydroxy compounds, and the sodium salt of ethylenediamine tetraacetic acid. All of these agents had some stabilizing effect. In the presence of EDTA no viscosity decrease occurred during storage, which suggests that trace metals act as catalysts in the oxidative degradation of Carbopol 934. The lower initial viscosity of the gels containing EDTA is due to the ionic deswelling which occurs when electrolytes are added to neutralized Carbopol 934 (1, 2). This incompatibility of the polymer with electrolytes prevents the use of ionic antioxidants such as sodium bisulfite. Nordihydroguaiaretic acid, propyl gallate, and ethyl hydrocaffeate had to be discarded as antioxidants since these compounds undergo a pronounced color change in the solutions of neutralized Carbopol 934.

TABLE I.—THE EFFECT OF DAYLIGHT ON THE VISCOSITY OF 0.7% NEUTRALIZED CARBOPOL 934 GELS

Neutralizing Agent	Initial Viscosity,[a] in c. p. s.	Viscosity after 17 days, in c. p. s.[b] Exposed to Daylight	Change %	Not Exp. to Daylight	Change, %
Sodium hydroxide	57,600	34,100	−41	58,900	+2
Triethanolamine	67,000	45,300	−32	69,200	+3
Ammonium hydroxide	57,200	28,000	−51	59,700	+4
Potassium hydroxide	56,400	37,500	−34	58,600	+4

[a] Average of eight samples of the sodium salt of Carbopol; four samples of the other salts.
[b] Average of four samples of the sodium salt of Carbopol; two samples of the other salts.

* Received October 10, 1957, from the University of California School of Pharmacy, San Francisco 22.
This study was conducted in part under a contract with the Armed Services Medical Procurement Agency.
† Present address: University of Buffalo School of Pharmacy, Buffalo 14, N. Y.
[1] A product of B. F. Goodrich Chemical Company, Cleveland, Ohio.

TABLE II.—THE EFFECT OF DAYLIGHT ON THE VISCOSITY OF 0.7% SODIUM CARBOPOL 934 GELS IN THE PRESENCE OF STABILIZING AGENTS[a]

Stabilizing Agent	Concentration, %	Initial Viscosity in c. p. s.	Viscosity, in c. p. s., after 17 days Exposure to Daylight	Change, %
Butylated Hydroxyanisole[b]	0.05	59,900	53,800	−10
Monothioglycerol[c]	0.5	56,100	54,100	− 4
Thiourea	0.5	59,800	56,300	− 6
Ethanol 95%	10.0	60,600	49,500	−18
Glycerin	10.0	64,300	55,600	−14
Propylene Glycol	10.0	61,200	53,200	−13
EDTA	0.05[d]	42,200	42,000	0

[a] Average of two samples. [b] "Sustane," Universal Oil Products Co., Chicago, Ill. [c] "Thiovanol," Evans Chemetics, Inc., New York, N. Y. [d] In terms of the acid form.

The results of this study show that pharmaceutical products containing neutralized Carbopol 934 as the thickening agent undergo significant viscosity breakdown when exposed to light. Such products must either be packaged in light-resistant containers or they must contain suitable stabilizing agents.

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The Formation and Structure of the Iron Salt of Benzyl Penicillin Procaine Using Infrared Spectroscopy*

By HERMAN A. SZYMANSKI and NICHOLAS PANZICA

THE FORMATION of coordination compounds may be studied using infrared spectroscopy. Valuable structural details may be obtained by analysis of the absorption bands produced by compound formation. This study was initiated to study the type of bonding and structure which is introduced when the metal iron is coordinated with benzyl penicillin procaine. Since this laboratory was also studying other simple coordination compounds, the results could be interpreted using the analysis suggested by these simple structures.

EXPERIMENTAL

Iron chloride solution was prepared by adding the reagent to anhydrous ethyl ether until a saturated solution was produced. Anhydrous sodium sulfate was added and the solution was mixed well and then centrifuged. Water content was checked using the Karl Fischer method (1). Iron content was determined by the 1,10 orthophenanthroline method (2). This solution was then stored in a tightly stoppered bottle containing a small amount of anhydrous sodium sulfate.

Benzyl penicillin procaine was obtained from the Charles Pfizer Company, Groton, Conn., and standardized at the Arner Company, Inc., Buffalo, N. Y. Microbiological assay was made using the method suggested in the Federal Register (3).

Benzyl Penicillin Procaine Iron.—This was prepared by several methods, the most successful of which was by dissolving the procaine in chloroform and adding ferric chloride solution described previously. Low temperature increased the yield. Ether was then added to form a precipitate and followed by filtration and washing with ether. Iron content, antibiotic activity relative to the starting material, melting point, and infrared spectra were then determined. The product was then redissolved in methyl alcohol and precipitated again, using ether, and the physical properties rechecked. The best analysis yielded an iron content of 4.3%, m. p. of 140–144°, and antibiotic acitivity of 105%.

Apparatus.—The infrared spectra were determined on a Baird-Atomic double beam model AB-2 spectrophotometer. Spectra were run in potassium

* Received November 6, 1957, from Canisius College, Chemistry Department, Buffalo 8, N. Y.
Supported in part by a grant from the Research Corporation, New York, N. Y.

bromide pellets, as mulls, and in several solvents.

Discussion of results.—The iron salt of benzyl penicillin sodium has been prepared and analyzed by Jadassohn (4). The iron analysis suggested the possibility of three moles of penicillin coordinating with one mole of iron. Calculations for the compound prepared in this work indicated that an iron content of 4.27% would be the theoretical per cent. This compares favorably with the 4.30% obtained for this compound.

The absorption at 5.6 μ for penicillin compounds has been correlated with antibiotic activity (5), a strong absorption indicating large activity. Figure 1 is the spectrum of the coordination compound and the high absorption at 5.6 μ agrees with the strong antibiotic activity of this compound.

The analysis of this spectrum has suggested that the peaks at 5.93, 7.87, and 8.50 indicate the procaine portion of the molecule and may be compared to those obtained for procaine hydrochloride and benzyl penicillin procaine (Figs. 2 and 3).

The 6.2 μ band in Fig. 3 is probably made up of the carboxylate C=O, phenyl group absorption and an absorption due to the procaine molecule. This band shifts on coordination compound formation, suggesting the iron may be forming an iron penicillinate.

The doublet at 4.0 μ in the coordination compound has been under intensive study by this group since we have obtained this same band with tertiary amine compounds of arsenic and antimony trichlorides. The same doublet appears in procaine hydrogen chloride and amine hydrochloride salts which have been determined in this laboratory. These results therefore suggest that the iron has produced a linkage of the type $(R\text{-}NH_3)_3$—Fe in this compound as a secondary linkage. The primary linkage being a bond between the iron and the acid group in the penicillin forming a penicillinate. Scale models of this proposed structure indicate the geometry would be satisfied by this structure.

Bands at 6.0 and 6.6 generally indicate monosubstituted amides while disubstituted amides show bands only at 6.0. In benzyl penicillin procaine as well as its iron compound, no 6.6 band occurs although most penicillins are monosubstituted amides. It is suggested that this band is missing due to hydrogen bond formation between the N-H normally

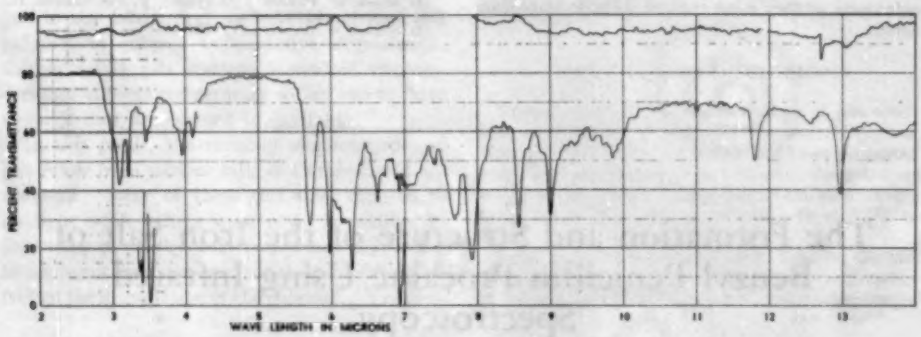

Fig. 1.—Infrared spectrum of the iron salt of benzyl penicillin procaine in potassium bromide.

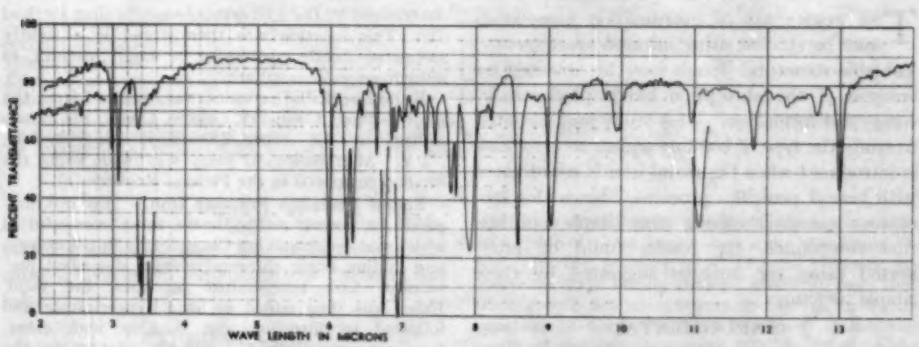

Fig. 2.—Infrared spectrum of procaine hydrochloride in potassium bromide.

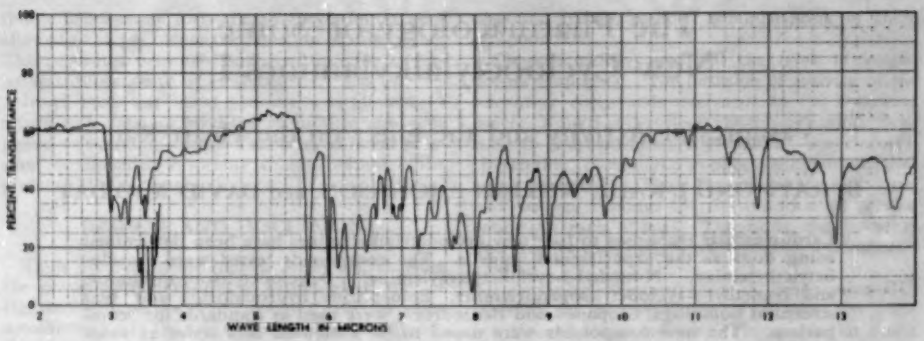

Fig. 3.—Infrared spectrum of benzyl penicillin procaine.

causing this absorption at 6.6 μ, and the carbonyl oxygen in the ester group of the procaine molecule. Similarly, the nonexistence of this peak in the iron salt suggests the procaine part of the molecule has not been disturbed by the iron coordination.

CONCLUSIONS

The ability to coordinate the spectra of simple compounds of similar structure to more complex ones, such as the compound reported here, has made it possible to assign a structure to the compound.

It is intended to further use this technique on other complex salts. An attempt was made to obtain the cobalt salt of benzyl penicillin procaine. The resulting material showed no antibiotic activity and its spectra suggested some breakdown had occurred. The spectra is shown in Fig. 4. The absence of a peak at 5.75 μ which is present in the spectra of benzyl penicillin procaine and its iron salt suggests that this complex has broken down, although the doublet at 4.0 μ is still present, suggesting some type of salt structure is present. Spectra of salts using ferrous iron and ferric salts of benzyl penicillin potassium are ill-defined and suggest considerable impurity present. This work will be continued and results will be reported in a later issue.

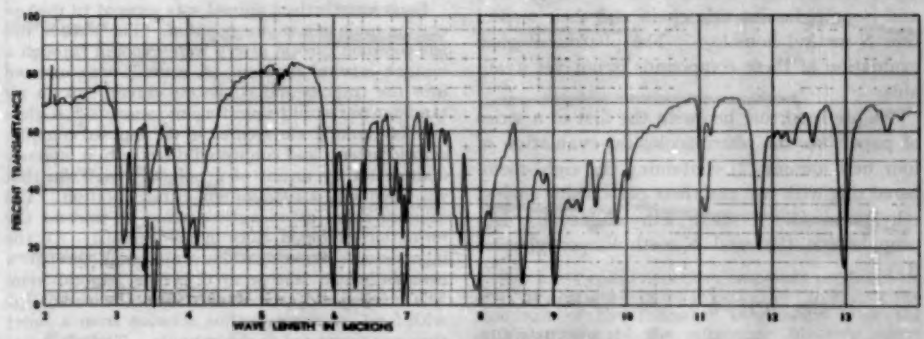

Fig. 4.—Infrared spectrum of benzyl penicillin procaine:Co.

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The Pharmacology of Some New Cycloöctylalkylamines I.*

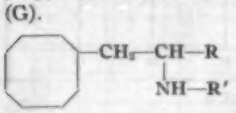
Vasopressor Activity and the Effect on the Heart Rate

By RAYMOND J. KAHL, WILLIAM E. JOHNSON, and DAVID W. O'DAY

Cardiovascular responses to four new cycloöctylalkylamines have been determined using dogs as the experimental animals. The compounds tested were β-cycloöctylethylamine, N-methyl-β-cycloöctylethylamine, β-cycloöctylisopropylamine, and N-methyl-β-cycloöctylisopropylamine. Epinephrine hydrochloride and two structural homologs, Clopane® and Benzedrex®, were used as standards for comparison. The new compounds were found to be somewhat less active as vasopressor agents than Clopane and Benzedrex when compared with epinephrine hydrochloride. All four cycloöctylalkylamines showed an increase in heart rate. This increase ranged from moderate to rather marked and was sustained for at least four minutes. The vasodepressor activity of the new compounds was found to be about the same as that of Clopane and Benzedrex when these compounds were injected following the administration of the adrenergic blocking agent Dibenamine.®

SINCE the appearance on the market of two cycloalkylalkylamines, namely, cyclopentamine, Clopane, containing a five-membered ring, and propylhexedrine, Benzedrex, containing a six-membered ring, an attempt has been made to further correlate cycloaliphatic ring size with sympathomimetic activity. McCarthy and Brown (1) prepared four compounds containing the seven-membered ring with the ethylamine and isopropylamine side chain and their respective N-methyl homologs. The pharmacological evaluation of these compounds is not yet available.

We are reporting herewith the first of a series of papers on the pharmacological evaluation of four new amines (2) containing the eight-membered ring with two and three-carbon side chains: ethylamine (D), N-methylethylamine (E), Isopropylamine (F), and N-methylisopropylamine (G).



D, $R = R' = H$

E, $R = H$, $R' = CH_3$

F, $R = CH_3$, $R' = H$

G, $R = R' = CH_3$

The work herein described dealt with the vasopressor activity of these cycloöctylalkylamines before and after the administration of the adrenergic blocking agent Dibenamine. The change in heart rate affected by the intravenous administration of these compounds was also recorded.

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The authors are indebted to Smith, Kline and French Laboratories of Philadelphia, Pa., for the Benzedrex® and to Eli Lilly Company of Indianapolis, Ind., for the Clopane® used in this study.

EXPERIMENTAL

Dogs that ranged from 6.6 to 16.4 Kg. in body weight and of random sex were used for blood pressure, respiration, and cardiac rate recordings. The animals were anesthetized with 45 mg./Kg. of intraperitoneal pentobarbital sodium. A total of 8 male and 15 female dogs were used in the determination of pressor responses to the new compounds and to the selected standards. Heart rate recordings were obtained on a total of 7 dogs.

Each anesthetized animal was secured to the operating table in a supine position. The trachea and left common carotid artery were exposed through a midline cervical incision. A cannula was inserted into the trachea and connected directly to an air tambour to record respiratory movements. A glass arterial cannula was secured in the exposed common carotid artery, and connected directly to a mercury manometer by means of a polyethylene tube that was filled with a 10% sodium citrate solution. The solution acted as an anticoagulant to prevent the formation of blood clots in the cannula. All the intravenous administrations were made through a cannula which was secured in the femoral vein. The compounds were washed into the circulation with 5 ml. of normal saline solution from a buret that was connected to this cannula. The heart rate was determined by means of a stethoscope and was recorded on a rapidly turning kymograph drum.

Epinephrine HCl in doses of 0.01 mg./Kg. was selected as a primary standard for comparison. It was administered at the beginning and at the end of each experiment to ascertain the reactivity of the animal. Clopane and Benzedrex were used as additional standards for comparison. They were administered in doses of 1.0 mg./Kg. calculated on the basis of the hydrochloride salt. The new amines were given in doses of 1.0 mg./Kg. calculated on the basis of the free amine but were administered as the hydrochloride salt.

Dibenamine in doses of 15 mg./Kg. was used as a sympathetic blocking agent to determine if the vasopressor activity of the new compounds could be reversed by such an agent. After the administra-

tion of Dibenamine, forty-five to sixty minutes were allowed to elapse before the injection of this amine. This procedure permitted sufficient time for a sympathetic blockade to be established.

In all cases the blood pressure was allowed to return to a constant level before another test compound was administered. This level of blood pressure corresponded favorably to the normal blood pressure of the animal.

RESULTS AND DISCUSSION

Table I contains the pressor responses, showing the elevations in pressure, as expressed in mm. of Hg, that were produced in dogs by the intravenous administration of the new compounds and the standards. It should be noted that all the compounds tested were less than 0.0072 times as active as pressor agents as was epinephrine HCl under the conditions of the experiments. The pressor activity of these compounds shows that they decrease in potency in the following order: epinephrine HCl, Clopane HCl, Benzedrex HCl, N-methyl-β-cycloöctylisopropylamine, β-cycloöctylethylamine, N-methyl-β-cycloöctylethylamine, and β-cycloöctylisopropylamine. Figure 1 shows kymograph recordings of typical pressor responses that were produced on dogs by the intravenous administration of these compounds.

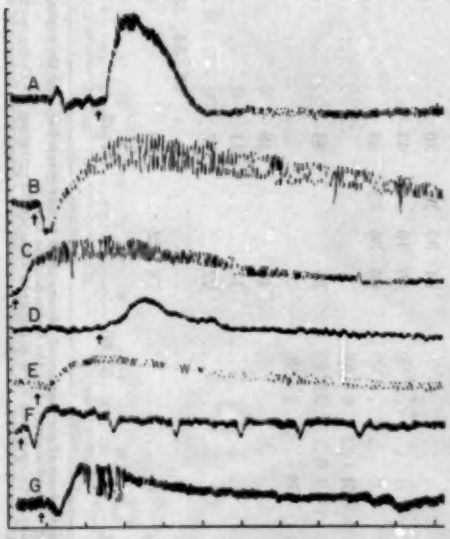

Fig. 1.—Typical pressor responses produced on dogs by the intravenous injection of the hydrochloride salt of the following compounds: A = Epinephrine HCl, 0.01 mg./Kg.; B = Clopane HCl, 1.0 mg./Kg.; C = Benzedrex HCl, 1.0 mg./Kg.; D = β-cycloöctylethylamine HCl, 1.0 mg./Kg.; E = N-methyl-β-cycloöctylethylamine HCl, 1.0 mg./Kg.; F = β-cycloöctylisopropylamine HCl, 1.0 mg./Kg.; and G = N-methyl-β-cycloöctylisopropylamine. Each division on the vertical scale equals 10 mm. of Hg, and each division on the horizontal scale is equivalent to one minute of elapsed time.

Clopane usually produced the most prolonged pressor responses. The duration of activity of the remaining compounds decreased in the following order: N-methyl-β-cycloöctylisopropylamine, Benzedrex, β-cycloöctylisopropylamine, β-cycloöctylethylamine, N-methyl-β-cycloöctylethylamine, and epinephrine HCl. Table II contains the individual and average readings for the duration of pressor action on each animal. The table also presents averages for each drug when tested on a number of animals.

Intravenous administration of Dibenamine HCl reversed the pressor action of all the new compounds and the standards. This particular phase of the evaluation was performed on a limited number of animals, and only a qualitative determination can be reported. Figure 2 contains kymograph recordings of depressor responses that were obtained by the administration of the test compounds after the administration of Dibenamine HCl.

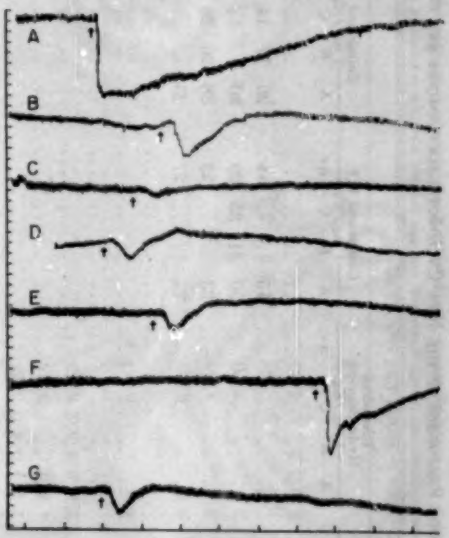

Fig. 2.—Typical depressor responses produced on dogs by the intravenous injection of the hydrochloride salt of the following compounds after the administration of the adrenergic blocking agent Dibenamine. A = Epinephrine HCl, 0.01 mg./Kg., B = Clopane HCl, 1.0 mg./Kg., C = Benzedrex HCl, 1.0 mg./Kg. D = β-cycloöctylethylamine HCl, 1.0 mg./Kg. E = N-methyl-β-cycloöctylethylamine HCl, 1.0 mg./Kg., F = β-cycloöctylisopropylamine HCl, 1.0 mg./Kg., and G = N-methyl-β-cycloöctylisopropylamine. Each division on the vertical scale equals 10 mm. of Hg, and each division of the horizontal scale is equivalent to one minute of elapsed time.

Respiratory responses to the 1.0 mg./Kg. doses of the new compounds were not uniform. Only

TABLE I.—COMPARATIVE PRESSOR RESPONSES OF THE NEW CYCLOÖCTYLALKYLAMINES AND THE SELECTED STANDARDS

Dog. No.	Sex	Epinephrine Hydrochloride 0.01 mg./Kg. A[a]	B	C	Av.	Clopane Hydrochloride, 1 mg./Kg. A	B	C	Av.	Benzedrex Hydrochloride, 1 mg./Kg. A	B	C	Av.	Compound D,[b] 1 mg./Kg. A	B	C	Av.	Compound E,[c] 1 mg./Kg. A	B	C	Av.	Compound F,[d] 1 mg./Kg. A	B	C	Av.	Compound G,[e] 1 mg./Kg. A	B	C	Av.
1	M	58	60	58	59	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
2	F	29	30	25	28	..	..	..	...	..	..	..	...	15	..	..	15	20	17	14	17	..	..	..	...	..	..	..	...
3	F	28	38	33	33	..	..	..	...	..	..	..	...	30	31	25	29	30	..	..	30	..	..	..	...	..	..	..	...
4	M	45	54	51	50	..	..	..	...	..	..	..	...	23	..	..	23	38	26	26	30	..	..	..	...	..	..	..	...
5	F	47	58	53	53	..	..	..	...	..	..	..	...	27	..	..	27	23	..	..	23	..	..	..	...	33	20	..	27
6	M	49	73	81	68	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	38	..	..	38
7	F	45	62	76	61	88	16	..	52	..	..	..	...	..	..	..	...	..	..	..	...	20	..	..	20	15	11	8	11
8	F	76	58	...	67	50	30	..	40	..	..	..	...	..	..	..	...	..	..	..	...	18	14	16	16	..	..	..	...
9	F	109	...	...	109	78	..	..	78	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
10	M	65	...	...	65	64	..	..	64	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
11	F	76	...	...	76	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	36	..	..	36
12	F	81	...	...	81	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	32	..	..	32	..	..	..	...
13	M	48	102	...	75	..	..	..	...	64	30	15	36	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
14	F	106	110	...	...	..	..	..	...	66	19	..	43	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
15	F	68	90	106	88	..	..	..	...	66	36	23	42	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
16	M	92	83	...	87	61	22	8	30	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
17	F	138	136	128	134	..	..	..	...	84	..	..	84	..	..	..	...	..	..	..	...	..	..	..	...	91	82	74	82
18	F	134	...	...	134	58	..	..	58	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
19	F	82	100	...	91	..	..	..	...	46	..	..	46	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
20	F	146	100	...	123	..	..	..	...	11	..	..	11	..	..	..	...	..	..	..	...	20	16	12	16	..	..	..	...
21	M	68	74	...	71	..	..	..	...	22	..	..	22	..	..	..	...	11	14	13	13	..	..	..	...	..	..	..	...
22	F	77	90	...	83	..	..	..	...	..	..	..	...	38	34	33	35	..	..	..	...	..	..	..	...	..	..	..	...
23	M	70	88	...	79	..	..	..	...	78	22	..	50	..	..	..	...	..	..	..	...	..	..	..	...	..	..	..	...
Average all Animals					74.8				53.66				41.7				27.8				22.6				21.0				38.8

[a] The letters A, B, and C denote successive doses of the compound administered to any given dog, and the average response is recorded in the column labeled Av. Doses of the new compounds were calculated on the basis of the free amines and were administered as the hydrochloride salts.
[b] Compound D = β-cycloöctylethylamine. [c] Compound E = N-methyl-β-cycloöctylethylamine. [d] Compound F = β-cycloöctylisopropylamine.
[e] Compound G = N-methyl-β-cycloöctylisopropylamine. The pressor responses are in terms of an increase in mm. of Hg over the normal blood pressure of the animal.

TABLE II.—DURATION OF PRESSOR ACTION OF THE NEW CYCLOÖCTYLALKYLAMINES AND THE SELECTED STANDARDS

Dog No.	Sex	Epinephrine Hydrochloride —0.01 mg./Kg.— A[b]	B	C	Av.	Clopane Hydrochloride —1 mg./Kg.— A	B	C	Av.	Benzedrex Hydrochloride —1 mg./Kg.— A	B	C	Av.	Compound D,[c] —1 mg./Kg.— A	B	C	Av.	Compound E,[d] —1 mg./Kg.— A	B	C	Av.	Compound F,[e] —1 mg./Kg.— A	B	C	Av.	Compound G,[f] —1 mg./Kg.— A	B	C	Av.
1	M	1.4[a]	3.2	1.9	2.17	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2	F	1.3	1.1	1.8	1.40	...	...	...	...	...	...	...	...	...	...	...	...	1.4	2.0	1.8	1.73	...	...	...	...	...	...
3	F	1.3	1.4	1.1	1.27	...	...	...	...	...	...	...	...	3.0	5.0	3.0	3.67	3.2	...	...	3.20	...	...	...	...	...	...
4	M	3.1	2.7	3.0	2.93	...	...	...	...	...	...	...	...	5.0	...	...	5.00	5.0	5.2	5.0	5.07	...	...	...	...	...	...
5	F	3.5	5.0	5.0	4.50	...	...	...	...	...	...	...	...	4.5	...	...	4.50	7.0	...	...	7.00	...	...	...	...	8.2	6.0 ... 7.10
6	M	1.0	4.5	5.0	3.50	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	9.0	3.5 ... 6.25
7	F	1.8	2.4	2.5	2.23	14.5	6.5	...	10.50	...	...	...	...	...	...	...	...	...	...	...	...	4.0	...	...	4.00	5.0	2.3 3.5 3.60
8	F	2.2	3.5	...	2.85	9.5	10.	...	9.75	...	...	...	...	...	...	...	...	...	...	...	...	5.0	6.5	5.5	8.50	...	...	...
9	F	4.0	...	...	4.00	8.5	...	...	8.50	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
10	M	1.3	...	...	1.30	8.0	...	...	8.00	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
11	F	1.2	...	...	1.20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	5.0	... ... 5.00
12	F	2.2	...	...	2.20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	3.7	...	...	3.70	...	...	...
13	M	0.9	1.5	...	1.20	...	...	...	...	7.7	4.0	4.0	5.23	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
14	F	1.2	1.2	...	1.20	...	...	...	...	4.3	3.0	...	3.65	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
15	F	1.2	1.5	2.0	1.57	...	...	...	...	7.0	6.8	5.8	6.53	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
16	M	1.4	2.3	...	1.85	2.7	1.2	0.7	1.53	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
17	F	2.2	3.0	3.0	2.73	...	...	...	...	8.0	...	...	8.00	...	...	...	...	...	...	...	...	...	...	...	...	8.3	9.6 9.7 9.20
18	F	1.6	...	...	1.60	1.3	...	...	1.30	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
19	F	3.0	3.6	...	3.30	...	...	...	...	8.0	...	...	8.00	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
20	F	1.5	...	...	1.50	...	...	...	...	3.6	...	...	3.60	...	...	...	...	...	...	...	...	3.5	3.5	4.0	3.67	...	...	...
21	M	1.6	1.7	...	1.65	...	...	...	...	2.0	...	...	2.00	...	...	...	...	1.5	2.0	1.3	1.60	...	...	...	...	...	...	...
22	F	1.5	1.7	...	1.60	...	...	...	...	...	...	...	...	4.5	4.0	2.5	3.67	...	...	...	...	...	...	...	...	...	...	...
23	M	1.3	...	...	1.30	...	...	...	...	5.2	3.2	...	4.20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Average all animals					2.13				8.55				5.15				4.21				3.72				4.97			6.23

[a] The figures represent the minutes required for the blood pressure to return to normal after each injection.

[b] The letters A, B, and C denote the successive doses of the compound administered to any given dog, and the average response is recorded in the column labeled Av.

[c] Compound D = β-cycloöctylethylamine, [d] Compound E = N-methyl-β-cycloöctylethylamine. [e] Compound F = β-cycloöctylisopropylamine. [f] Compound G = N-methyl-β-cycloöctylisopropylamine.

minor variations in the respiratory rate and amplitude could be detected.

The figures in Table I reveal a striking difference in the tachyphylaxis produced by the cycloöctylalkylamines when compared with Clopane and Benzedrex. The inability of subsequent doses to produce a response approximating the initial pressor reaction was much more pronounced in the case of Clopane and Benzedrex.

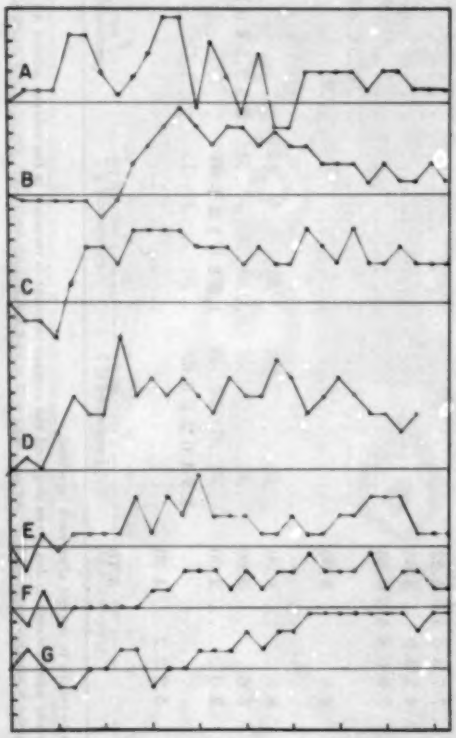

Fig. 3.—Changes in heart rate produced on dogs by the intravenous injection of the standards and the cycloöctylalkylamines. The dose in each case was 1.0 mg./Kg., except epinephrine HCl which was 0.01 mg./Kg. A = Epinephrine HCl, B = Clopane HCl, C = Benzedrex, D = β-cycloöctylethylamine, E = N-methyl-β-cycloöctylethylamine, F = β-cycloöctylisopropylamine, and G = N-methyl-β-cycloöctylisopropylamine. The horizontal lines across the figure represent the normal heart rate in each case. Displacement of the curves above and below these lines represent an increase or decrease in heart rate respectively. Each division on the vertical scale represents a change in heart rate of 5 beats per minute. Each division on the horizontal scale at the bottom of the figure equals an elapsed time of thirty seconds.

Heart rate changes that resulted from the administration of the evaluated compounds are graphically presented in Fig. 3. β-Cycloöctylethylamine was found to be the most potent cardiac accelerator of the new compounds. Clopane and Benzedrex were slightly less potent. The remaining three cycloöctylalkylamines were considerably less active than any of the other compounds that were tested.

SUMMARY

1. Certain cardiovascular responses to four recently synthesized sympathomimetic amines (N-methyl-β-cycloöctylethylamine, β-cycloöctylethylamine, N-methyl-β-cycloöctylisopropylamine, and β-cycloöctylisopropylamine) have been evaluated on dogs. Epinephrine HCl, Clopane HCl, and Benzedrex HCl were used as standards for comparison.

2. The compounds can be listed in order of decreasing pressor potency as epinephrine HCl, Clopane HCl, Benzedrex HCl, N-methyl-β-cycloöctylisopropylamine, β-cycloöctylethylamine, N-methyl-β-cycloöctylethylamine, and β-cycloöctylisopropylamine.

3. When listed in order of decreasing duration of action, the compounds are arranged as Clopane HCl, N-methyl-β-cycloöctylisopropylamine, Benzedrex HCl, β-cycloöctylisopropylamine, β-cycloöctylethylamine, N-methyl-β-cycloöctylethylamine, and epinephrine HCl.

4. The sympathetic blocking agent, Dibenamine HCl, reversed the pressor action of all the new compounds and the standards.

5. The new compounds produced no significant changes in respiratory rate and amplitude in the 1.0 mg./Kg. doses that were used.

6. The new compounds produced considerably less tachyphylactic effect on blood pressure than did Clopane HCl and Benzedrex HCl.

7. In 1.0 mg./Kg. doses the compounds produced cardiac accelerator activity in the following order of decreasing potency: β-cycloöctylethylamine, Clopane and Benzedrex, N-methyl-β-cycloöctylethylamine, N-methyl-β-cycloöctylisopropylamine, and β-cycloöctylisopropylamine. In 0.01 mg./Kg. doses epinephrine HCl was about equal in cardio-accelerator potency to the 1.0 mg./Kg. doses of Clopane and Benzedrex.

REFERENCES

(1) McCarthy, W. C., and Brown, T. H., THIS JOURNAL, 43, 661(1954).
(2) McCarthy, W. C., and Kahl, R. J., *J. Org. Chem.*, 21, 985(1956).

Tragacanth Solutions I.

The Relation of Method of Preparation to the Viscosity and Stability*

By GERHARD LEVY† and T. W. SCHWARZ

Various factors which influence the viscosity of tragacanth solutions are discussed. The effects of homogenization and heat on solutions of the gum are reported. The degree of initial hydration of the gum is related to the viscosity changes that occur during aging. This viscosity may first increase and then decrease, or it may solely decrease. Studies of the viscosity changes that occur over relatively long periods of time show that the magnitude of such changes may be predicted by methods which are outlined in this paper.

TRAGACANTH is the dried gummy exudation from Asiatic species of *Astralagus* (Fam. *Leguminosae*). It is a hydrophilic colloid of carbohydrate nature (1, 2). One of the oldest gums known to man, it was described by Theophrastus several centuries before Christ; and it has been official in every edition of the United States Pharmacopeia since 1820.

Tragacanth has a long history and even today it is one of the most important gums used in pharmacy. It is employed as a thickening, suspending, and stabilizing agent in jellies, water-soluble ointments, emulsions, suspensions, and mucilages. It is used in ephedrine sulfate jelly N. F. X, in methylrosaniline chloride jelly, N. F. IX, in thimerosal jelly N. F. IX, and in a large number of nonofficial products. The fact that six out of the seven contraceptive jellies listed in New and Nonofficial Remedies 1955[1] include tragacanth as a thickening agent, is an indication of its wide acceptance in industry. The present-day importance of the gum is evidenced by two recent reports from the laboratories of major pharmaceutical houses (3, 4) which use it as the thickening and suspending agent in new drug formulations.

Despite the time-tested qualities of tragacanth, it shares some of the disadvantages inherent in the use of all natural gums. It is a relatively crude natural product, whose chemical constitution and thickening capacity are influenced by climatic changes and methods of harvesting. Consequently, products that contain tragacanth as the thickening agent may vary in consistency and in aging characteristics (5–10).

Numerous investigators have reported that the viscosity of tragacanth solutions is influenced by the method of preparation, pH, and storage temperature. The gum appears to be composed of two different constituents, one soluble in water, the other swelling but not dissolving in water (11). Many investigators found that the viscosity of tragacanth solutions increases markedly with time (1, 7–10, 12). Chambers (13) attributed this viscosity increase to a slow process of hydration. He regarded a recently prepared solution as being only partially hydrated, consisting of incompletely hydrated globules, which may be likened to the discontinuous phase of a two-phase system dispersed in a continuous phase of dissolved and hydrated material. In time, some of the swollen particles of the discontinuous phase go into solution, while the already dissolved gum slowly undergoes degradation (14).

The two processes, hydration and degradation, which occur simultaneously during aging, result in viscosity changes that have been difficult to predict and which have made the commercial preparation of tragacanth solutions with uniform properties a somewhat specialized and empirical endeavor.

Since most freshly prepared tragacanth solutions are only partially hydrated, their viscosity will rise as hydration proceeds. When the "depot" of nonhydrated gum is exhausted, no further viscosity increase will occur. Simultaneously, the hydrated gum undergoes degradation. As more gum goes in solution, it too undergoes degradation. Thus the viscosity decrease due to breakdown gradually exceeds the viscosity increase due to continuing hydration. When the rate of hydration equals that of degradation, the viscosity of the solution remains constant; and during this phase of the aging process the viscosity will be at its maximum. After tragacanth is fully hydrated, the viscosity begins to decrease markedly because the con-

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This paper is adapted from the prize-winning manuscript submitted by Gerhard Levy in the Western Region, 1957 Lunsford Richardson Pharmacy Awards Competition.
† Present address: University of Buffalo School of Pharmacy, Buffalo 14, N. Y.
[1] Subsequent editions of N. N. R. did not list the composition of the jellies.

tinuing degradation of the gum is no longer offset by further hydration.

This study was undertaken to attempt the evaluation of the factors that contribute to the hydration as well as to the degradation of tragacanth in aqueous solution. Solutions in which the gum is completely hydrated yet little decomposed should exhibit the highest viscosity. Thus, if the degree of hydration could be regulated without causing simultaneous degradation, it should be possible to prepare solutions in which the following properties may be controlled and adjusted to the specific requirements of the product: (*a*) initial viscosity; (*b*) maximum viscosity which the preparation will attain; (*c*) the time at which the viscosity is fairly constant: whether initially, or after varying periods of storage; (*d*) the length of time during which a minimum effective viscosity is maintained.

These properties are of great importance in pharmaceutical formulation. For this reason, the investigation aimed at developing a method for obtaining maximum hydration of tragacanth with little or no simultaneous degradation. Two main approaches were followed: homogenization (mechanical treatment) and application of heat.

In addition, accelerated aging tests were carried out on tragacanth solutions prepared by different methods in order to obtain further knowledge about the effect of such procedures on the long-term viscosity changes of the gum.

EXPERIMENTAL

Preparation of Samples.—All samples were prepared with tragacanth gum, U. S. P. ribbon No. 1. The solutions were prepared by adding the gum to 200-ml. portions of preserved water (methylparaben, U. S. P., 0.1%, propylparaben, U. S. P., 0.025%, distilled water *q. s.*) contained in an eight-ounce wide-mouth glass jar. The samples were then agitated by a mechanical shaker for twenty-four hours.

Viscosity Measurement.—Samples of the tragacanth solutions were placed in 50-ml. centrifuge tubes and occluded air was removed by centrifugation at 2,000 r. p. m. for ten minutes. The samples were then brought to a temperature of 25° ± 0.5° in a constant-temperature bath. The viscosity was determined with the Brookfield Synchro-Lectric Viscometer, Model LVF, using spindle No. 4 at 6 r. p. m. Readings were taken after ten minutes of spindle rotation.

Homogenization.—Homogenizations were carried out with a single-piston hand homogenizer[1] or with a Charlotte Colloid Mill, Model A. The clearing in each piece of equipment was kept constant: the nozzle on the hand homogenizer was kept one turn short of being completely tightened and the clearance on the colloid mill was adjusted to 0.0025 inches.

Stability Test.—Stability tests were made by storing the samples in eight-ounce glass jars at a temperature of 50° ± 1°. Samples were removed periodically and their viscosity was determined after storing for twenty-four hours in a water bath at 25°.

All experiments were carried out in duplicate and the data in the graphs are average values.

RESULTS AND DISCUSSION

The Effect of Homogenization.—A 1.5% tragacanth solution prepared two days earlier was passed repeatedly through a hand homogenizer. Samples were collected after each passage and the viscosity of these samples was determined after twenty-four hours, as previously described.

Another solution of 1.5% tragacanth was passed repeatedly through a colloid mill. Samples were collected after each passage and their viscosity was determined twenty-four hours later. The results are shown in Fig. 1.

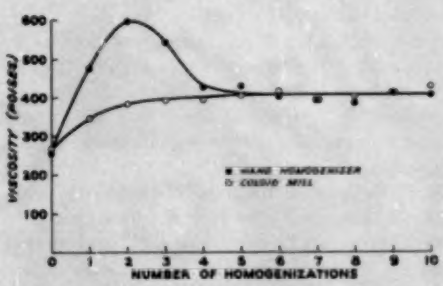

Fig. 1.—The effect of homogenization on the viscosity of a 1.5% tragacanth solution.

The graph indicates the viscosity increase of the tragacanth solutions as a result of homogenization. Both solutions eventually reached the same viscosity plateau, where additional passages through the hand homogenizer or the colloid mill caused no further viscosity change. It is significant that the solution which was passed through the hand homogenizer reached a viscosity peak before attaining the constant lower viscosity level.

The effect of homogenization in increasing the viscosity of tragacanth solutions has been previously reported (13, 15) but as far as the authors could establish, this is the first instance in which the attainment of a viscosity plateau after repeated homogenizations is described.

It is important to note that the solution which underwent treatment with a hand homogenizer reached the same viscosity plateau as the solution which was subjected to shear by the colloid mill.

The homogenization of tragacanth solutions causes a reduction in the size of the dispersed globules of partially hydrated gum. The increased surface area exposed to the water brings about a

[1] Distributed by the Central Scientific Company, Chicago 13, Ill.

greater hydration of the gum and as a result the viscosity of the solution is increased. It has been found previously and follows from basic concepts of colloid chemistry that, for example, milk and clay suspensions increase in viscosity as a result of homogenization. Philippoff (16) attributes this effect to a better solvation of the particles as a consequence of particle size reduction. It is likely that the same considerations apply to the effects of the homogenization of tragacanth solutions.

The presence of the hump in the viscosity curve of the solution which was passed through the hand homogenizer introduces an interesting problem of interpretation on the nature of the internal structure of tragacanth solutions. Philippoff (16) states that mechanical treatment of gel-forming substances may cause a decrease in their viscosity. Such a decrease may be partly reversible and partly irreversible. He distinguishes between "labile" structural viscosity and "stable" structural viscosity. The former is sensitive to mechanical treatment while the latter is not.

The hump, which is evident in the viscosity curve of the solution which was passed through the hand homogenizer, may be an indication of "labile" structural viscosity. Several passages through the hand homogenizer were required to completely destroy this "labile" component. On the other hand, the absence of the viscosity hump in the solution which was passed through the colloid mill may be attributed to the much more severe mechanical treatment which that solution underwent. Evidently, the labile component was completely destroyed by the first passage through the colloid mill.

Considering the chemical nature of tragacanth, a compound of high molecular weight containing many polar groups, it is possible that mechanical treatment may cause a rupture of hydrogen-bonds which bind the gum molecules to each other. Once such bonds are broken, water molecules may replace gum molecules so that individual gum molecules are bound to water alone instead of to each other. After the "labile" viscosity component was thus destroyed, only the "stable" viscosity component remained, which apparently is not affected by the mechanical treatment and which accounts for the viscosity plateau.

The Effect of Heat.—The effect of subjecting tragacanth solutions to graded amounts of heat in the course of their preparation is shown in Fig. 2. In this experiment, beakers containing 200 ml. of preserved water were placed in a boiling water bath. When the preserved water began to boil, the tragacanth was added and the solution was agitated with an electric stirrer. After boiling the solutions for the indicated periods, they were removed from the water bath and permitted to come to room temperature. The water lost by evaporation was then replaced, the solutions were agitated in a mechanical shaker for two hours, and placed in a water bath at 25°. On the next day, the viscosity of the solutions was determined in the usual manner.

The graph shows that boiling for periods longer than fifteen minutes caused a considerable decrease in the viscosity of the solutions, which indicates more pronounced degradation than hydration. Since at least thirty minutes of boiling were required to dissolve the tragacanth ribbons, it is obvious that the application of sufficient heat to expedite solution results in considerable degradation of the gum.

Tragacanth solutions subjected to graded amounts of heat were repeatedly passed through the hand homogenizer as previously described. Their viscosity was determined after each homogenization. The results are presented in Fig. 3. The solutions that had been heated for the longest period of time had the lowest viscosity plateau. Their initial viscosity hump also became less evident and disappeared entirely in the sample that was heated for sixty minutes.

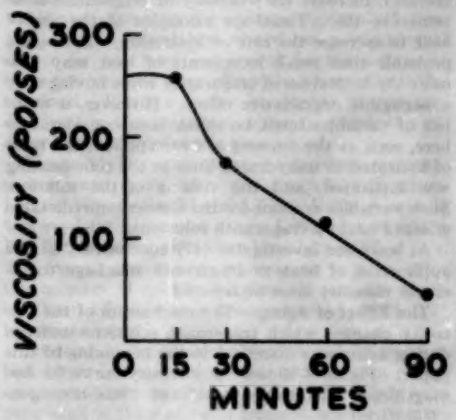

Fig. 2.—The effect of heat (100°) used in the preparation of 1.5% tragacanth solutions on their viscosity.

These observations illustrate the effect of heat on both the "labile" and "stable" structural viscosity of tragacanth. Heat has probably three effects: it causes degradation of the gum, it ruptures hydrogen bonds, and it increases the rate of hydration of tragacanth. Obviously, the lowering of the viscosity plateaus in the heat-treated solutions reflects the known chemical degradation of the gum by heat.

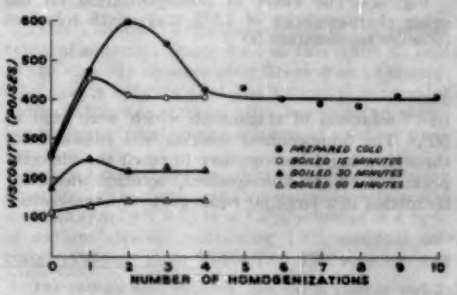

Fig. 3.—The effects of heat and homogenization on the viscosity of 1.5% tragacanth solutions.

Gralen and Karrholm (14) were able to show this degradation by determining the average molecular weight of tragacanth in solution before and after boiling. They report an average molecular weight change from 840,000 to 550,000 in the samples which they had boiled for thirty minutes. After boiling a tragacanth solution for three hundred and thirty minutes, the average molecular weight was reduced to 350,000.

The rupture of hydrogen bonds between gum molecules by heat is probably responsible for the decreasing prominence of the viscosity hump.

Some reports appearing in the literature state that short periods of boiling, usually two to fifteen minutes, increase the viscosity of tragacanth solutions (14–16). These are examples of the use of heat to increase the rate of hydration. It is quite probable that small increments of heat may promote the hydration of tragacanth while having only a negligible degradative effect. However, a number of variables must be taken into consideration here, such as the amount of heat applied, the ratio of hydrated to unhydrated gum at the time heating was instituted, and the volume of the solution. Such variables account for the almost unpredictable effect of heat on tragacanth solutions.

At least one investigator (17) concluded that the application of heat to tragacanth mucilage to increase viscosity must be rejected.

The Effect of Aging.—The mechanism of the viscosity changes which tragacanth solutions undergo during aging was discussed in the beginning of this paper. Figure 4 shows the viscosity curve for homogenized, nonhomogenized, and "half-homogenized" solutions of tragacanth which were kept at 50°. The homogenized solution was passed twice through a hand homogenizer to reach the viscosity peak. The "half-homogenized," solution was made by mixing in a large jar equal parts of homogenized and nonhomogenized gum. The graph indicates that the viscosity of the homogenized solution, after a very small initial increase, showed very little change for about two weeks. After twenty-one days, the viscosity began to decrease rapidly. Since the initial viscosity of the solution showed very little further increase, it can be assumed that the gum was almost completely hydrated by homogenization.

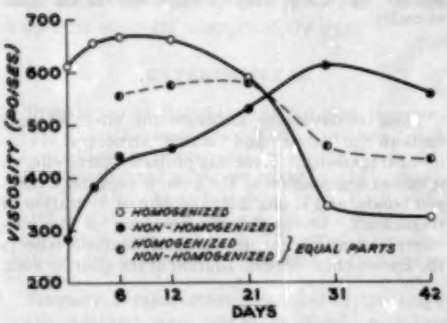

Fig. 4.—The effect of homogenization on the aging characteristics of 1.5% tragacanth solutions (storage temperature 50°).

The viscosity of the nonhomogenized gum increased with time as expected. It reached a maximum in thirty-one days at 50°. From then on the viscosity began to decrease.

The "half-homogenized" solution illustrates how controlled partial homogenization may be used to obtain intermediate viscosity effects. The magnitude of the viscosity changes that occurred in the test period was much smaller with the "half-homogenized" gum than with the other two solutions.

These observations suggest that methods for preparing aqueous tragacanth solutions may be specifically designed to obtain products of different stability characteristics.

Solutions intended for relatively short storage periods may be completely homogenized. Such solutions will show no significant viscosity gain and little viscosity decrease for some time.

Solutions intended for long periods of storage may be prepared by dissolving the tragacanth in cold water without subsequent homogenization. Such solutions increase in viscosity on aging. A viscosity drop occurs only after relatively long periods of time. The approximate maximum viscosity which such solutions can reach, may be predicted by homogenization of a sample.

Solutions intended for intermediate periods of storage may be partially homogenized. The viscosity changes of such solutions may be regulated by varying the ratio of homogenized to nonhomogenized gum in accordance with previously stated considerations.

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The Role of Residual Calcium in the Viscosity Changes of Sodium Alginate Solutions*

GERHARD LEVY† and T. W. SCHWARZ

The marked viscosity increase of most sodium alginate solutions after freezing and thawing is due to and proportional to the amount of residual calcium present. The rheological character of such solutions is also altered as a result of freezing. Sequestering agents and polyols affect the initial viscosity and modify the effects of freezing.

THE AUTHORS have recently reported (1) that solutions of a type of sodium alginate exhibit a marked viscosity increase after freezing and thawing. This behavior, which is apparent in most commercial types of sodium alginate, was found to be absent in highly purified material, which had a very low calcium content (2). This report presents the results of an investigation of the role of residual calcium in the viscosity changes of sodium alginate solutions, especially after freezing and thawing.

The major manufacturing processes for sodium alginate involve the precipitation of calcium alginate by addition of a soluble calcium salt such as calcium chloride to the crude algin. The calcium alginate is subsequently converted to alginic acid and then to sodium alginate (3, 4). The sodium alginate of commerce contains varying amounts of residual calcium, depending, among other factors, on the number of washings of the alginate with hydrochloric acid solution and the origin of the kelp. Since the severity of the extraction process required to obtain highly purified sodium alginate reduces the thickening capacity and increases cost, most commercial products contain appreciable amounts of residual calcium.

EXPERIMENTAL

Materials.—Five types of pharmaceutical grade sodium alginate obtained from three different manufacturers were used in this study. Since most manufacturers produce several different types of sodium alginate, no further reference to the source is made.

Methylparaben and propylparaben were U. S. P. grade, while the remaining chemicals were of reagent grade.

Preparation of Solutions.—Solutions of sodium alginate were prepared by sprinkling the powder on 80% of the required amount of preserved water (0.1% methylparaben and 0.025% propylparaben in distilled water) agitated in a Waring Blendor. The solutions were sheared at a definite rate for ten minutes.

Any other component of the solution was dissolved in 20% of the required amount of preserved water and added to the sodium alginate solution. The samples were then placed on a mechanical shaker for one hour and stored for three days at room temperature after which the viscosity was determined. When a buffer was used, it was predissolved in the preserved water to which the sodium alginate powder was then added.

Viscosity Determinations.—Viscosity measurements were made with the Brookfield Synchro-Lectric Viscometer, Model LVF. A No. 4 spindle was used for viscosities above 5,000 c. p. s. and a No. 3 spindle for viscosities less than 5,000 c. p. s. The measurements were made at six r. p. m. after ten minutes of spindle rotation on samples which had been adjusted to a temperature of 25° ± 0.5° in a water bath.

Freezing.—The solutions were stored for twenty-four hours in the freezing compartment of a refrigerator at a temperature of −16° ± 1°. They were permitted to thaw at room temperature for twenty-four hours and placed in a water bath at 25° for two hours before the viscosity was again determined.

Calcium Determination.[1]—Calcium determinations were made with a flame spectrophotometer similar to one described by MacIntyre (5). Calcium absorption was measured at 423 mμ in order to avoid interference by sodium. All samples were previously digested with sulfuric and nitric acids. The calcium content of sodium alginate is reported in terms of the dried powder.

Loss on Drying.—The procedure used was that of the National Formulary, Tenth Edition, for sodium alginate.

Per cent concentration of sodium alginate is reported here, however, in terms of "wet" weight, i. e., before drying.

RESULTS AND DISCUSSION

Table I shows the calcium content of the various types of sodium alginate used in this study as well as the viscosity changes after freezing and thawing. The results indicate that the viscosity increase of sodium alginate solutions after freezing and thawing is proportional to the amount of calcium present.

Figure 1 represents the effect of adding increasing amounts of sodium ethylenediamine tetraacetic acid solution (pH 8.3) to a 1.3% solution of a type of sodium alginate containing 1.3% residual calcium[2] (Product A in Table I). The solution was

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This study was conducted in part under a contract with the Armed Forces Medical Procurement Agency.
† Present address: University of Buffalo, School of Pharmacy, Buffalo 14, N. Y.

[1] The authors wish to thank Drs. G. S. Gordan and F. Loken of the University of California Medical School for the calcium determinations.
[2] The two identical figures of 1.3% are coincidental.

TABLE I.—THE EFFECT OF RESIDUAL CALCIUM ON THE VISCOSITY CHANGES OF SODIUM ALGINATE SOLUTIONS AS A RESULT OF FREEZING AND THAWING

Product	Solution w/v, %	—Per cent Calcium Content— Manufacturer's Data[a]	Experimental[b]	Loss on Drying,[d] %	—Viscosity in c. p. s.[c]— Before Freezing	After Freezing	Ratio: After/Before
A	2	1.5	1.3	8.7	32,700	87,000	2.7
B	2	1 to 1.5	1.0	7.5	25,900	56,200	2.2
C	3	0.5	0.55	14.6	44,750	78,600	1.8
D	4	0.2	0.15	12.2	42,800	44,200	1.03
E	3	"Low Ca"	0.098	13.7	24,400	24,000	0.98

[a] Approximate data; *A* and *D* in terms of CaO. [b] As Ca^{++} based on dried alginate. [c] Average of four samples. [d] Average of two determinations.

adjusted to a pH of 8.3 with diethylbarbiturate buffer of 0.1 *M* total concentration to maintain constant sequestering capacity. The arrow on the graph indicates the point at which all the calcium should be sequestered, as calculated on the basis of the assayed calcium content.

The results show that solutions of sodium alginate below a minimal concentration of unsequestered calcium undergo no viscosity increase due to freezing, and that EDTA can be used to prevent such viscosity changes of sodium alginate solutions containing more than the minimal amount of residual calcium. The addition of sodium citrate or sodium hexametaphosphate instead of EDTA produced the same effect.

Figure 2 illustrates the effect of increasing concentrations of calcium acetate on the viscosities of a 2% solution of a type of sodium alginate containing only 0.098% residual calcium (Product *E* in Table I). While small amounts of added calcium have practically no effect on the viscosity before freezing, they cause a considerable viscosity increase after freezing and thawing.

It appears that the calcium residue in sodium alginate is present partly as calcium alginate and partly in the form of soluble salts. Gomez (6) was able to remove part of the calcium by dialysis, indicating the presence of calcium not bound to alginate. On the other hand, the presence of calcium in the form of calcium alginate is indicated by the decrease in viscosity with the addition of increasing amounts of EDTA in sodium alginate solutions containing residual calcium (Fig. 1). This can only occur if the chelating agent substitutes sodium for the calcium which is bound as alginate. The result of this substitution is a decrease in the degree of cross-linking, which is characteristic of calcium alginate.

The viscosity change cannot be ascribed to an electroviscous effect since the concentration of buffer in the solution was sufficient to obliterate this effect. A further support for this assertion is that the addition of EDTA to a solution of highly purified, unbuffered sodium alginate (0.03% Ca) caused no viscosity change whatsoever.

As solutions of sodium alginate undergo freezing and pure ice separates from the solution, the effective concentration of calcium present in soluble form is increased. The equilibrium

$$2\ \text{Na alginate} + Ca^{++} \rightleftharpoons \text{Ca (alginate)}_2 + 2\ Na^{+}$$

is shifted to the right according to the mass law. This increase in calcium alginate concentration causes a greater degree of cross-linking which, in turn, results in a higher viscosity of the solution.

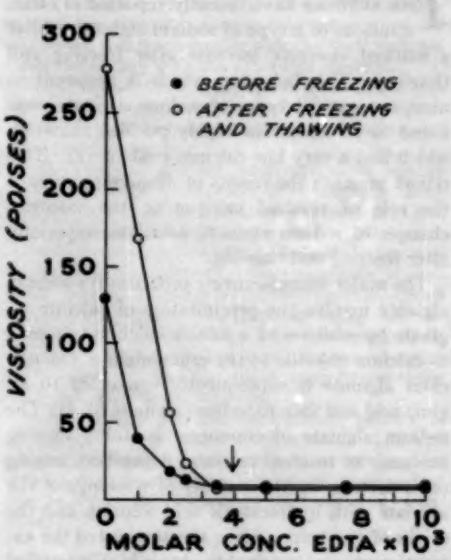

Fig. 1.—The effect of EDTA on a 1.3% solution of sodium alginate containing 1.3% residual calcium. (Average of three samples.)

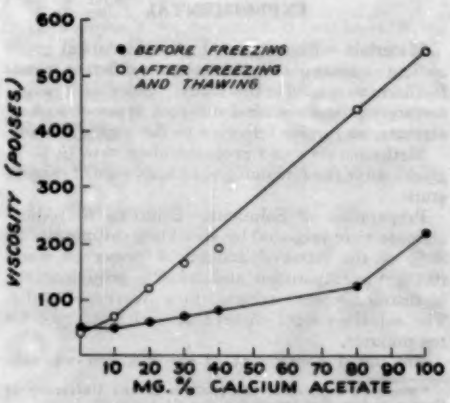

Fig. 2.—The effect of added calcium on a 2% solution of sodium alginate of low calcium content (0.098%). (Average of four samples.)

After thawing, there is a gradual reversal to the previous equilibrium, but this takes place at a very slow rate (1).

A further indication that the viscosity increase as a result of freezing is due to a concentration of calcium can be seen in the effect which variation of freezing temperature has on the viscosity increase. A solution of sodium alginate which exhibited a viscosity increase of 94% after freezing at −16° had a viscosity increase of only 28% after freezing at −2.5°, a temperature at which less ice will crystallize out of solution.

In a previous paper (1), it was shown that the presence of glycerin or propylene glycol in sodium alginate solutions containing residual calcium resulted in a smaller or no viscosity increase after freezing and thawing; also, that sodium alginate solutions containing large amounts of added calcium salt showed a negligible viscosity change after freezing and thawing. In both cases there was an increase in the initial viscosity which made any effect of freezing less noticeable. The presence of the hydrophyllic polyols results in a partial dehydration of the sodium alginate, probably followed by greater association of the alginate molecules with each other through hydrogen bonds. Furthermore, the presence of polyols lowers the freezing point and; therefore, the enrichment of calcium in the solution due to separation of ice is less than in the absence of polyols, provided the temperature is the same.

In the case of sodium alginate solutions containing large added amounts of calcium, sufficient calcium alginate is formed to give a high degree of cross-linking of alginate molecules. Thus freezing would have little or no further effect on the viscosity.

The formation of calcium alginate in a solution of sodium alginate changes not only the viscosity but also the rheological characteristics of the solution. While solutions of pure sodium alginate behave like pseudoplastic liquids (7), the presence of calcium alginate in such solutions produces a thixotropic system (8).

The freezing of sodium alginate solutions containing residual calcium causes a twofold change: the ratio of calcium alginate to sodium alginate is increased; and an essentially pseudoplastic system becomes markedly thixotropic. Although the Brookfield Viscometer does not lend itself to a quantitative measurement of thixotropy, it was possible to perceive the presence of a thixotropic component by the decrease in the apparent viscosity as a result of prolonged spindle rotation.

It should be pointed out that the marked viscosity increase of sodium alginate solutions, containing residual calcium, upon freezing and thawing cannot be considered a thixotropic sol-gel transformation. Thixotropic transition must be isothermic, yet the sol-gel change of the solution requires freezing. The change is actually a chemical transformation of part of the sodium alginate to calcium alginate, which imparts the characteristic rheological behavior of the latter to the system. Any interpretation of the viscosity data reported here must take into account this change in rheological properties.

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Quantitative Determination of a Series of Malonic Esters by Gas Chromatography*

By J. B. TEPE and H. J. WESSELMAN

Gas chromatography offers a convenient, rapid, and accurate method for analyzing a number of malonic esters which previously were determined less accurately by other physical methods.

FOR MANY YEARS, the production of malonic esters was controlled by means of specific gravity or refractive index measurements. While these methods are reasonably satisfactory for pure compounds or a binary mixture, they fail when applied to mixtures containing three or more components.

With the advent of gas chromatography, it was found highly desirable to apply this technique as a control in the production of malonic esters.[1] Using this method, a mixture of six esters can be determined simultaneously. This technique was first introduced by James and Martin (1) and has been used extensively and applied to a wide range of compounds. Books by Phillips (2) and Keulemans (3) give a very good discussion of the field of gas chromatography.

EXPERIMENTAL

Apparatus.—Gas Chromatograph.—Burrell Kromo-Tog, Model K-2. Column.—150 x 0.5-cm. glass column with integral heating coil. Column

* Received November 29, 1957, from Eli Lilly and Company, Indianapolis, Ind.
[1] The authors wish to thank Mr. R. W. Bridges for preparing pure samples of the malonic esters used in this work, and Mr. L. D. Seay for assaying the standard mixture of malonic esters.

packed with 20% (w/w) Linde Silicone Oil L-46 (12,500 Centistokes) on Celite C-44857 and operated at 220°. Carrier Gas.—Helium at an inlet pressure of 8 lb./sq. in. and atmospheric pressure at outlet. Flow rate 50 ml./min. Pipette.—Agla Micrometer Syringe, Sample size, 0.01 cc.

Pure samples of the esters to be studied were prepared by laboratory fractional distillation. These samples were chromatographed on various stationary phases. Of these, petrolatum, tri-*m*-cresyl phosphate, and silicone oil proved satisfactory; however, the relatively high temperature (220°) necessary for a good separation caused the petrolatum and tri-*m*-cresyl phosphate to gradually bleed from the column. The silicone column proved to be very stable at this temperature and more than 3,000 samples have been analyzed with this column.

The retention times of the pure esters were determined and a mixture of equal volumes of each ester was then used to calibrate the instrument before the production lots of the various esters were analyzed.

Results are easily reproduced when the operating conditions of the instrument remain constant. In routine analysis, it has proved advantageous to run the standard sample along with each lot of esters being analyzed.

PROCEDURE

The sample to be analyzed is injected into the column through a rubber diaphragm by means of a micrometer syringe. After the sample has passed through the column and the thermal conductivity cell, the chart is removed from the recorder and the amount of each component is calculated. A typical chart for a mixture of six malonic esters is shown in Fig. 1.

Since the retention time for each ester is specific under a given set of experimental conditions, each ester can be determined qualitatively very easily. Similarly, the area under each peak is proportional to the amount of that constituent in the mixture. Whether this area is proportional to weight per cent or mole per cent is still controversial. In actual practice, this discrepancy can be eliminated by calibrating the system, using either weight per cent or mole per cent.

The areas under the peaks can be measured with a planimeter, by cutting out the peak and weighing, by coupling an integrator to the detector output, or by measuring the peak heights by half-bands widths

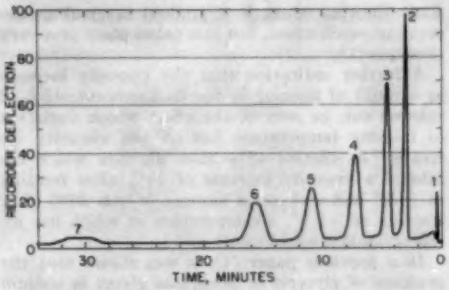

Fig. 1.—Retention times of six malonic esters using silicone column at 220°. 1—Air, 2—Diethyl malonate, 3—Diethyl ethyl malonate, 4—Diethyl diethyl malonate, 5—Diethyl isoamyl malonate, 6—Diethyl isoamyl ethyl malonate, 7—Diethyl diisoamyl malonate. Sample, 0.01 cc.

(3). When area is used for calculation of relative composition with the assumption that the malonic esters have the same thermal conductivity, the equation becomes:

$$\frac{\text{Area of Individual Peak}}{\text{Sum of Areas of All Peaks}} \times 100 = \% \text{ Individual Component in Mixture}$$

Table I shows the results of three separate assays of the standard mixture of malonic esters. Diethyl diisoamyl malonate is not included in this assay as it is not manufactured and does not appear in chromatograms of the production lots of esters.

SUMMARY

A mixture containing several malonic esters can be assayed qualitatively and quantitatively by means of gas chromatography. Without this method, the assay of such a mixture is tedious and time consuming, in addition to being inaccurate at its best.

TABLE I.—TYPICAL ANALYSIS OF A STANDARD MIXTURE OF MALONIC ESTERS

	Area Per cent				
Run No.	Diethyl Malonate	Diethyl Ethyl Malonate	Diethyl Diethyl Malonate	Diethyl Isoamyl Malonate	Diethyl Isoamyl Ethyl Malonate
1	11.0	12.2	19.0	22.6	35.0
2	12.8	13.4	18.9	21.5	33.4
3	12.7	12.2	18.9	22.8	33.4
Average	12.2	12.7	18.9	22.3	33.9
Theory weight, %	12.6	12.1	17.8	23.1	34.4
Theory volume, %	11.8	11.8	17.6	23.5	35.3
Theory mole, %	17.1	14.0	17.9	22.0	29.0

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Plant Microtechnique: A Note On Rapid Tissue Dehydration*

By ARNOLD C. NEVA

TO THE STUDENT and the research worker, current tissue microtomy in many instances presents sharp contrasts of methods. The standard reference works on microtechnique largely perpetuate the time-consuming conventional methods of dehydration, embedding, and mounting of tissues predominantly for descriptive studies. Conversely, and with noticeably greater frequency during the past decade, the current literature presents a variety of differing techniques, adaptations, improvements, and innovations to both operational functions and intent.

Recurrent emphasis ranges from automatic sectioning and mounting (1, 2) and chelate application (3); to histochemical (4), and rapid one-step dehydration and infiltration operations (5); and to dynamic bioactivity and tissue changes rather than the structural make-up and organization of tissues.

Because modern microtechnique demands an ever increasing number of studies with a minimum of undesirable solvent effects, greater simplification in procedures is the prevailing trend. This also holds true for a better interpretation of structure and organization in tissues as well.

For example, the rapid animal tissue dehydration method with modified Soxhlet extractor, as reported by Saiki and Kling (6), sought to circumvent the time-consuming serial tissue transfer in graded alcohol dilutions and other varients of this long used process.

Without elaborating on initial application trials that will be dealt with in other subsequent detailed reports, the method of Saiki and Kling was not found to be fully applicable to plant tissue work. But with several modifications, the basic principle of rapid tissue dehydration, as outlined by these authors, has been satisfactorily applied to plant tissues.

This time-saving rapid tissue dehydration process can easily be carried out by the following steps, briefly described in the following sequence, and by using whatever modifications may be necessary in individual cases.

The plant material is cut to suitable (approx. 1 square cm.) size and aspirated for approximately one-half hour, or somewhat longer if necessary. Aspiration is carried out in a solution made up of: alcohol, 70 parts; glacial acetic acid, 15 or 10 parts; and water, 20 or 15 parts. Higher glacial acetic acid concentrations may be necessary with tougher woody plant materials; and conversely, it may not be necessary to use the acid at all when working with very soft plant tissues, or ordinary animal tissues.

By successive rinsings, avoiding air inclusion, the aspiration fluid is replaced completely with anhydrous acetone, the dehydrating liquid.

In contrast to the apparatus of Saiki and Kling, a regular unmodified Soxhlet extraction apparatus is employed with the material for dehydration in acetone placed in a regular extraction thimble loosely covered with cotton. If circumstances necessitate, a suitable variation for a tissue holder can be improvised that prevents the complete solvent run-off from the tissues; if for any reason, this may be objectionable. No difficulty has been encountered thus far in this respect by the writer.

The necessary volume of acetone is poured into the extraction chamber and after assembling, a sufficient excess is added to permit satisfactory continuous operation, and to compensate for any loss of acetone through volatilization.

Approximately 200 Gm. of Drierite[1] is placed into either a 500-cc. or 1-liter receiving flask to serve as a dehydrating agent. The extraction unit is assembled with the receiving flask, and with a cooling condenser. Only enough heat is used to volatilize the acetone, and the dehydration is carried out by the regular Soxhlet extraction procedure.

Satisfactory dehydration of plant tissues is usually accomplished within a two to three-hour period, although certain difficultly dehydrated substances may take somewhat longer.

After dehydration, the anhydrous acetone is replaced with xylol or any of the other regularly used annydrous solvents; and the microtechnique process is continued with the conventional paraffin embedding, or with other similar methods as desired.

Fresh plant tissues have been satisfactorily processed into paraffin for sectioning in one day by the method described.

This significant saving of time is not the only advantage of rapid tissue dehydration achieved, although it is the principal one. For example, interesting histochemical fixations have resulted in studies using the acetone rapid dehydration method. Certain cell content precipitations and localizations, the nature of which is yet undetermined, have been witnessed in a particular leaf tissue. Also, just as other workers have used this solvent to good advantage in previous enzyme histochemical techniques, acetone could well be tried more generally for work in tissue culture techniques and in other enzyme histochemical studies.

Another advantage of rapid tissue dehydration, not particularly because of the solvent employed, is the retention of easily loosened and obliterated delicate structures gained by the minimum amount of handling of the material.

By employing several diverse types of plant materials that continue to be the subjects of further expanded studies, the rapid tissue dehydration method has been found suitable under the conditions described and should be applicable for most microtechnique tissue processes.

* Received April 17, 1956, from Duquesne University, School of Pharmacy, Pittsburgh 19, Pa.

The writer wishes to express grateful appreciation for the earlier reviews of this report by the late Dr. Hugh C. Muldoon, Dean Emeritus, Duquesne University School of Pharmacy, and by Dr. Franklin A. Neva, Department of Tropical Public Health, Harvard School of Public Health, Boston, Mass.

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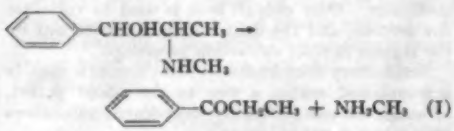
[1] W. A. Hammond Drierite Company's anhydrous calcium sulfate.

A Note on the Detection of Ephedrine by Spot Test*

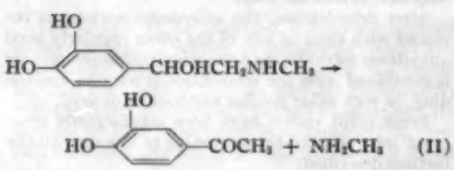
By FRITZ FEIGL and ERNESTO SILVA

SPOT TEST METHODS are not only just as reliable as the macromethods given in pharmacopeias for the identification of medicinal and pharmaceutical preparations, but also possess the great advantage of saving material, time, and effort. This fact has been mentioned previously (1) and methods for identifying certain compounds which had not previously been noted in the pharmacopeias were presented. As a supplement to this work, a new and quite specific test for ephedrine is described below, which is of interest in the testing of pressor substances (sympathomimetic amines).

Since alkali easily causes an hydramine cleavage of ephedrine and adrenaline according to the equations:



$$C_6H_5CHOHCHCH_3 \rightarrow \text{(NHCH}_3\text{)} \quad C_6H_5COCH_2CH_3 + NH_2CH_3 \quad (I)$$



$$HO\text{-}C_6H_4\text{-}CHOHCH_2NHCH_3 \rightarrow HO\text{-}C_6H_4\text{-}COCH_3 + NH_2CH_3 \quad (II)$$

Tests for these two amines have been described (2), which are based on the detection of the monomethylamine produced. The limits of detection by the spot test technique are 5γ of ephedrine and 8γ of adrenaline. When ephedrine alone is to be detected by the hydramine cleavage, the preliminary removal of adrenaline is necessary. This is easy to achieve, since ephedrine is very soluble in ether, while adrenaline is completely insoluble. In dealing with acid solutions of chlorides, sulfates, etc., one must, therefore, shake first with alkali and then with ether.

It has been found that the hydramine cleavage of ephedrine, can be detected by making use of its behavior on pyrolysis in the presence of oxidizing agents. If ephedrine hydrochloride is evaporated to dryness in the presence of alkali and the dry residue heated over an open flame after being mixed with MnO_2, PbO_2, Pb_3O_4, Mn_3O_4, or Co_3O_4, acetaldehyde is split out. This is apparently the result of the oxidation of the phenylethyl ketone produced by the hydramine cleavage, according to the reaction:

$$C_6H_5COC_2H_5 + 2O \rightarrow C_6H_5COOH + CH_3CHO \quad (III)$$

Schmidt (5) has found that phenylethyl ketone is split out on dry distillationo of chlorides of ephedrine and pseudo-ephedrine in a stream of CO_2. In accord with this, and with the oxidation according to (III), is the fact that ephedrine mixed only with excess CaO or MnO_2 and heated, gives off acetaldehyde, although only in small quantities. The acetaldehyde formed by this pyrolytic oxidation can be identified in the gas phase by the test of Lewin (4) (blue color formation in a piperidine-sodium nitroprusside solution).

Dry heating of ephedrine or its salts with sodium bismuthate ($NaBiO_3$) has proved most suitable for performing the decomposition to acetaldehyde and for using it analytically. This basic compound of pentavalent bismuth acts simultaneously as an alkalizer to catalyze the hydramine cleavage and as an oxidizing agent for the phenylethyl ketone produced. The acetaldehyde is detected with a morpholine-sodium nitroprusside solution (3).

Adrenaline in centigram quantities does not produce any acetaldehyde, and hence it may be distinguished from ephedrine absolutely.

Procedure.—A minimal amount of the solid substance to be tested, or of the dry residue from a drop of a solution of the base or its salts is mixed with several centigrams of sodium bismuthate in a micro test tube. A piece of filter paper which has been moistened with a drop of the aldehyde reagent is placed over the open end of the tube, and the bottom is heated with a microflame. A positive test is shown by the formation of a circular blue spot on the yellow reagent paper. The intensity of the color depends on the quantity of ephedrine.

When ephedrine is to be detected in very dilute solution it is best to put some glass powder into the tube, and to evaporate a drop of the test solution on this powder, after which the sodium bismuthate is added and mixed in. In this way one obtains good contact for the pyrolytic oxidation. Limit of detection: 5γ ephedrine.

Reagent.—A freshly prepared mixture of equal volumes of 20% water solution of morpholine and 5% solution of sodium nitroprusside in water was used.

It may be expected that Ephetonal (*p*-amino compound of ephedrine) will equally split off acetaldehyde on pyrolytic oxidation.

The user of the test described above should note that compounds with $-OC_2H_5$ and $>NC_2H_5$ groups also yield acetaldehyde on dry heating with sodium bismuthate, but in smaller amounts than does ephedrine.

* Received September 10, 1957, from the Laboratorio da Producao Mineral, Ministerio da Agricultura, Rio de Janeiro, Brazil.

Translated by Peter Oesper, Hahnemann Medical College, Phila., Pa.

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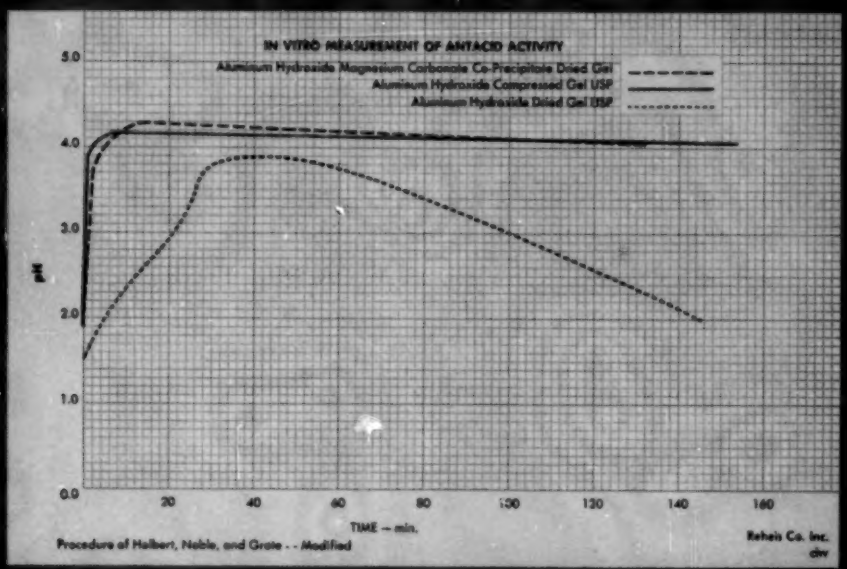

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